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THE EFFECTS OF FORAGE TYPE AND ANTAGONISTS ON COPPER METABOLISM IN SHEEP

By

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ABSTRACT

Three *in vivo* and two *in vitro* experiments were carried out to investigate the effect of forage type and dietary antagonists molybdenum (Mo) and sulfur (S) on Cu metabolism in sheep. In experiment 1, dried grass pellets or maize silage without or with added dietary Mo and S were fed to Texel growing lambs to investigate the effect of forage type and antagonists on Cu status and performance. The maize silage fed lambs had a higher weight gain and rumen pH, but a lower liver Cu concentration compared with the dried grass pellets fed lambs. The addition of antagonists significantly reduced liver Cu status, but blood Cu parameters were not affected by dietary treatment. In experiment 2, grass haylage vs. maize silage were used to investigate the effect of forage type and antagonists on Cu metabolism in Swaledae growing lambs. The maize silage fed lambs had a higher weight gain and liver Cu status but a lower rumen pH compared with the grass haylage fed lambs. Liver Cu status, Pl-Cu concentration, and Cp activity were decreased by the inclusion of Mo and S, while Cp:Pl-Cu ratio was not affected by antagonists. In experiment 3, the involvement of the rumen digesta fractions on Cu metabolism in forages used in experiment 1 and 2 plus grass silage was investigated. Cu, Mo, and S were found mainly (above 85%) associated with the solid phase of the fermented rumen digesta, at the expense of supernatant fraction. Additional Mo and S significantly reduced Cu distribution in the supernatant fraction due to increasing Cu incorporation into the solid phase. In experiment 4, the effect of forage preservation on rumen pH and their interaction between Cu and antagonists were investigated. Rumen pH in grass silage fed lambs tended to be lower compared with lambs fed other forages. Lambs fed urea and fermented WCW were heavier than lambs fed grass silage. Liver Cu status was higher in lambs offered urea WCW or grass silage compared with fermented WCW. Additional antagonists substantially reduced liver Cu status, but had a small effect on blood Cu parameters. No effect of Cu antagonists were observed on liver Cu retention in lambs fed fermented WCW, whereas they significantly reduced liver Cu retention in lambs on urea WCW and grass silage. Blood Cu parameters were slightly affected by dietary treatment. In experiment 5, the effect of preservation of fresh grass as hay or silage on Cu distribution in rumen fluid following *in vitro* fermentation was investigated. Preservation of fresh grass as hay or silage had no effect of Cu distribution in the fermented rumen liquor or after pepsin-HCl digestion. These series of studies showed the effect of forage type on Cu metabolism. In addition, it confirms that dietary Mo and S are potent Cu antagonists and this potency may be reduced at a lower acidic rumen environment.

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Previous appeared work

Part of the work in this thesis has appeared previously:

Hussein, A.A., Mackenzie, A.M., Wilkinson, R.G. and Huntington, J.A. 2016. The effects of forage type and molybdenum and sulfur supplementation on copper status in growing lambs. *Advances in Animal Biosciences*, (7) 1, pp. 72.

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Declaration

The work in this thesis is original. None of this work has been presented in any previous application for a degree.

Abdulqader Hussein

Chapter 1. Literature Review

1.1. Introduction

There are 22 minerals that have been shown to be essential in an animals' diet in order to maintain normal health and production (Suttle, 2010). These minerals, based on the amount required by the animal, are separated into 7 major or macro elements and 15 trace or micro elements (Table 1.1) (Underwood, 1981). Among trace elements only copper, iodine manganese, zinc, cobalt, and selenium have been required to be supplemented into the diet of ruminant livestock in order to prevent their deficiency (Suttle, 2010). Furthermore, dietary supplements of other minerals such as aluminium, boron, cadmium, lithium, lead, and rubidium have also been shown to improve growth and health, but are not classed as essential (Underwood and Suttle, 2004).

Table 1.1. Essential macro and micro elements in animals' diets

Macronutrient	Micronutrient
Calcium (Ca)	Iron (Fe)
Potassium (K)	Iodine (I)
Phosphorous (P)	Zinc (Zn)
Sodium (Na)	Copper (Cu)
Chlorine (Cl)	Manganese (Mn)
Magnesium (Mg)	Cobalt (Co)
Sulfur (S)	Molybdenum (Mo)
	Selenium (Se)
	Chromium (Cr)
	Tin (Sn)
	Vanadium (V)
	Fluorine (F)
	Silicon (Si)
	Nickel (Ni)
	Arsenic (As)

The essentiality of dietary Cu has long been established when it was shown to be required for growth and haemoglobin formation in laboratory rats (Elvejhem *et al*., 1929). Copper was also found to be essential for preventing diseases that are naturally caused in grazing animals such as diarrhoea in cattle in Somerset, UK (Ferguson *et al*., 1943). In addition, subsequent studies demonstrated that Cu was an essential component for numerous enzymes and proteins such as tyrosinase, ceruloplasmin, cytochrome c oxidase, lysyl oxidase, superoxide dismutase, and dopamine β-monooygenase (Suttle, 2010). Subsequently, the importance of the interaction between Cu and Mo was found when Cu was used as a treatment to control the diarrhoea ''teart'' caused in cattle grazing herbage rich in Mo (Ferguson *et al*., 1943). Similarly, Mo was used as a means to treat Cu toxicity in sheep fed herbage low in Mo concentration (Dick and Bull, 1945).

The development of these two syndromes not only depends on the concentration of Cu in the diet, but also on the concentration of Mo and a third element S (Dick, 1954). A three-way interaction between Cu, Mo, and S has been reported to be the main factor involved in the aetiology of clinical Cu deficiency disorders in ruminants (Suttle, 1991). The signs of clinical Cu deficiency are shown as swayback, reduced weight, decreased reproduction, alteration in wool characteristics, reduced feed intake, anaemia, cardiovascular disorders such as rupture of the aorta or heart failure, and impaired immune response (McDowell, 1985; NRC, 2005; Suttle, 2010). In ruminants, Cu deficiency can be either primary due to the presence of inadequate Cu in the diet, or secondary due to a presence of high levels of Cu antagonists minerals that reduce its availability and functions (Phillippo *et al*., 1987a; 1987b; Suttle, 1991). This review will discuss the nutritional and biological importance of Cu in ruminants. In addition, it will also discuss the difference in Cu availability between forages and interaction between forage type and mineral composition.

1.2. Properties of copper

1.2.1. Physical and chemical properties of copper

Copper has an atomic number of 29 and an average atomic weight of 63.546 daltons, belonging to the first transition metal group in the periodic table. Copper can be mostly found in the environment in one of the three oxidation states: Cu^{o} (copper metal), Cu^{1+} (cuprous ion) or Cu^{2+} (cupric ion) (Georgievskii *et al*., 1982). In the biological system, including water, Cu can be found mainly in cupric form (Cu^{2+}), and rarely in cuprous form Cu^{1+} (Linder and Maryam, 1996). The Cu^{1+} compounds are readily oxidised to Cu^{2+} in aqueous solution and Cu^{2+} compounds are the most oxidised state of Cu (NRC, 2005). Therefore, Cu is commonly found in compounds Cu^{2+} (Linder, 1991).

1.2.2. Dietary source of copper

Copper is a natural trace element which is widely distributed in feedstuffs (Table 1.2). Sources rich in Cu are whole grains, legumes, seeds, nuts, and by products. Grasses tended to have lower Cu content compared with legumes, and Cu content in leaves and stems tended to be lower than grains (Minson, 1990; McDowell, 1992).

Table 1.2. Dietary copper concentration of animal feedstuffs

Dietary source	Copper content (mg/kg DM)
Grass (Close grazing)	8
Grass (Extensive grazing)	7
Grass silage	3.2-10.8
Maize silage	2- 6.4
Alfalfa haylage	5.0-10
Hay	3.7-15.9
Straw	2-5.6
Soya	21.3
Barley	3.7-15.9
Rolled oats and barley	5.3-61.5
Maize grain	12-52
Sugar beet pulp	3.2-6.1
Molasses	5.7-15
Soya bean meal	25

Sources: McDowell (1992), Nicolson *et al*. (1999), and Li *et al*. (2005).

1.2.3. Factors affecting copper status of forages

The copper concentration in herbage varies with soil type, geographical location, plant species, plant parts, stage of maturity, and climate or season (Mison, 1990; MacPherson, 2000; Suttle, 2010). The ability of soil to provide Cu to animals via plant uptake depends on factors such as the level of Cu in the soil, water logging, and soil pH (Minson, 1990). The level of Cu in soils has been shown to be variable. For example, the total concentration of Cu was varied in four different Scottish soils such as olivine-gabbro (41 mg/kg DM), Sepernite (15 mg/kg DM), sandstone (8.5 mg/kg DM), and granite (7 mg/kg DM) (Burridge *et al*., 1983). In addition, the soil Cu concentration may in turn be affected by water irrigation and water logging, as freely drained soils such as basic igneous contained a higher Cu concentration compared with poorly drained soils (40 and 10 mg/kg respectively) (Burridge *et al*., 1983). Consequently, the Cu uptake by plant will be low if soil Cu concentration is low. Reddy *et al*. (1981) reported that the concentration of Cu in clover (*Trifolium*. subterranean) grown on a lateritic podsolic soil that contained 2.1 mg Cu/kg soil or in calcareous sand that contained 0.5 mg/kg soil was 12.9 and 6 mg/kg DM respectively.

The application of lime in order to improve upland pastures has generally produced little effect on Cu uptake by plants due to a rise in soil pH. However, it is nevertheless important in the aetiology of Cu deficiency in the sheep grazing in hill pastures, as the uptake of Mo was greatly increased by liming and the Cu:Mo ratio will be changed (MacPherson, 2000). Increasing soil pH from approximately 5.5 to 6.5 via liming resulted in a decreased Cu concentration in barley from 3.5 to 3.2 mg/kg DM, whereas, Mo concentration in red clover was increased from 1.4 to 4.7 mg/kg DM (Burridge *et al*., 1983).

The mean level of Cu in grass pasture in Shropshire (UK) has been found to be between 9.18 and 9.53 mg/kg DM, whereas, in another area such as Northumberland (UK) the level of Cu ranged from 6.33 to 11.43 mg/kg DM (Peers and Phillips, 2011). This difference may be related to soil type and it can be useful to determine areas where the risk of Cu deficiency is low or high (Jumba *et al*., 1995). Copper content among grass species, grown on the same soil, has been found to vary widely, ranging from 4.5 to 21.1 mg/kg DM (Suttle and Underwood, 1991). Minson (1990) reported that temperate grasses tended to contain less Cu compared to legumes in the same conditions (4.7 vs. 7.8 mg/kg DM, respectively) (Table 3.1).

The distribution of Cu in temperate grasses varies (Table 3.1); leaves on average contain 35% more Cu than the stem fraction, but this is affected by the age of the plant, with little difference between stem and leaves in immature plants (Minson, 1990). The maturity of plants leads to a reduced Cu level of forages as a consequence of a decreased proportion of leaves and lowering of the Cu concentration of the stem (MacPherson, 2000). The concentration of Cu in young oat plant *(Avena sativa)* was 9.4 mg/kg DM and reduced to 3.2 mg/kg DM at milk-ripe stage (MacDonald and Wilson, 1980). Seasonal change can cause some changes in Cu concentration in plants possibly due to the difference in soil temperature, as it is reported that increasing soil temperature from 12 to 20°C resulted in increased Cu concentration in clover plants (*Trifolium. subterraneum*) by 20-93% (Reddy *et al*., 1981). Likewise, Cu concentration in forage samples collected from three grass field and one red clover in spring was lower than in autumn (6.33 and 11.43 mg/kg DM respectively) (Peers and Phillips, 2011).

Table 1.3. Copper distribution between plant parts

Species		Plant part (mg/kg)	
Grasses		leaf	Stem
	Dactulis glomerat	7.1	5.4
	Festuca pratensis	4.9	3.5
	Lolium perenne	5	4
	Phleum pratense	4.6	3.2
Legumes			
	Lotus corniculatus	9.8	7.6[a]
	Medicago sativa	10.5	7.9[a]
	Trifolium repens	10.5	6.9[a]

[a]including petiole.

Source; Minson (1990).

1.3. Copper deficiency

Copper deficiency has usually been related to a low Cu status (Suttle, 2010) and can be termed ‘’hypocuprosis’’. There are many factors that have been related to Cu deficiency such as diet, breed, species, and Cu antagonists (Suttle, 2010). Copper deficiency in ruminant animals occurs when Cu levels in the diet are not sufficient to maintain optimum growth, health, and productivity (Underwood and Suttle, 2004). Copper deficiency can occur as either a primary deficiency, due to inadequate amounts of Cu in the diet, or as a secondary deficiency which is caused by interactions between Cu and one or more antagonists that reduce its availability or function (Phillippo *et al*., 1987a; 1987b). The Cu antagonists that have been shown to have the most significant effects are Mo, S, and Fe, and secondary Cu deficiency is generally more common and economically important as it may result in neonatal ataxia, depigmentation, altered keratinisation, growth retardation, infertility, disease susceptibility, or diarrhoea, and ataxia (MacDowell, 2003; NRC, 2005; Suttle, 2010).

Ataxia, also referred to as ‘swayback’ is a neurological disorder affecting lambs or kids from Cu-deficient pregnant ewes and is characterised by hind limb staggering, lack of coordinated movement, associated by low Cu levels in the brain, and liver (Ivan *et al*., 1990; Alley *et al*., 1996). Types of ataxia may occur in neonatal lambs which are completely paralysed or ataxic at birth and followed by death (Woolliams *et al*., 1986b). The delayed ataxia which is recognised by spastic paralysis, uncoordination of the hind legs, stiff and staggering gait, swaying hind quarters, often triggered by flock disturbances. The third is not common and occurs in older lambs and is characterised by a transfixed stance, head quivering, and sometimes blindness (Suttle, 2010). In other ruminant species nerve disorders, such as hind limb ataxia, have been reported in moose, and in red and fallow deer in the United Kingdom, New Zealand, and Sweden (Barlow *et al*., 1964; Wilson *et al*. 1979; Audigé *et al*., 1995). The level of the cytochrome *c* oxidase (COX) was found to decrease in the brain mitochondria of affected lambs’ (Smith *et al*., 1976; Alleyne *et al*, 1996). The main abnormalities in the central nervous system in cases of swayback result from demyelination, with associated reduction of COX activity (Prohaska, 1981), along with a decrease in dopamine production due to reduced dopamine monoxygenase activity (O’Dell *et al*., 1976). Demyelination is associated with degeneration of the motor neuron activity in brain and spinal cord in the lambs (Suttle, 1988). The vulnerability of lambs to neonatal ataxia has been associated with severe Cu deficiency in ewes in mid pregnancy, when a rapid phase of myelination in the fetal central nerve system (CNS) occurs (Suttle, 2010). Delayed ataxia is related to the deprivation of ewes in late pregnancy, when the second phase of spinal cord myelination occurs a few weeks after birth (Suttle, 2010).

A deficiency of Cu either as primary (Suttle *et al*., 1970) or secondary (Suttle and Field, 1968; Kendall *et al*., 2000; Majak *et al*., 2004), can cause some abnormalities in wool and hair. An early sign of Cu deficiency in sheep is often associated with lack of crimp in the wool fibre staple (Suttle and Angus, 1976) caused by an alteration in the keratinization process through reducing the cross linkage of disulfide bonds (Denks *et al*., 1972), which are linked polypeptide chains of keratin fibres formed by the oxidation of –SH groups of the cysteine residues, between polypeptide chains (Linder, 1991). These signs have been induced in experiments by adding high levels of Mo and S (Kendall *et al*., 2000) or by rearing Scottish blackface lambs on improved pastures (Whitelaw *et al*., 1977). Copper supplementation can quickly restore these abnormalities in new wool growth (Underwood, 1977).

Achromotrichia or loss of the hair or wool pigment is considered as the earliest or sometimes only sign of Cu deficiency (Suttle, 2010) which is caused by insufficient activity of the Cu containing enzyme tyrosinase, an enzyme involved in melanin pigment biosynthesis (Seo *et al*., 2007). White wool develops in normally black-woolled sheep, greying of black or bleaching of brown hair are most obvious signs of depigmentation (Underwood, 1977; Suttle, 2010). Also, in the Aberdeen Angus cattle, a brownish tinge can be seen in the coat and the skin becomes mottled (Hansen *et al*., 2009).

Effects of Cu deficiency on growth and performance have not always given consistent results. Whitelaw *et al*. (1979) and Woolliams *et al*. (1986b) demonstrated that the reduction in growth rate in sheep grazing on improved pasture was due to Cu deficiency. Similarly, Whitelaw *et al*. (1984) also reported the same effect in cattle. This reduction in growth rate was counteracted by Cu supplementation (Whitelaw *et al*., 1987b). Phillippo *et al*. (1987a; 1987b) also reported that hypocupraemic cattle had a reduced growth rate, as a result of dietary Mo but attributed this to a reduction in DMI. However, Williams (2004) and Sefdeen *et al*. (2016) did not observe any effect of dietary Cu antagonists on growth. The effect of antagonists on intake may be due to the effect of absorbed thiomolybdate that may have a direct effect on Cu containing enzymes such as peptidylglycin α–amidating monooxygenase, which exerts an influence on appetite-regulating hormones gastrin and cholecystokinin (Suttle, 2010).

Sub fertility in ruminant animals has been linked with clinical Cu deficiency in an early report by Murno (1957) suggesting a possible effect of Mo on fertility. Several authors also have reported the impact of the secondary Cu deficiency on sub fertility, including embryonic loss

(O'Grman *et al*., 1987), delayed onset of puberty (Phillippo *et al*., 1987a), decreased release or production of luteinised (LH) hormone or follicle stimulated hormone (FSH) (du Plessis *et al.*, 1999a), and lack of signs of behaviour in sheep (du Plessis *et al.*, 1999b). In the field, supplementation of Cu successfully improved molybdenum-induced subfertility in cattle (Black and French, 2000; Kendall *et al*., 2001). Mackenzie *et al.* (2001) also reported that poor conception in dairy cattle was attributed to the dietary Mo concentration in the diet and they showed that bolusing cattle resulted in a significant decrease in inseminations to confirmed conception from 2.5 to 1.7 compared with the control animals.

Early observation of diarrhoea in cattle grazing on the Mo rich 'teart' pasture in Somerset and in British Colombia were shown to be prevented by Cu supplementation (Ferguson et al., 1943). However, Ward *et al*. (1978) suggested that the diarrhoea may be a manifestation of Mo toxicity rather than its effects on Cu metabolism. Anaemia has been reported in association with severe or prolonged Cu deficiency (Suttle and Field, 1968; 1969; Whitelaw *et al*., 1979). Copper is required in ferroxidase enzymes to mobilise iron for haemoglobin synthesis (Suttle, 2010). In lambs, anaemia has been identified as either hypochromic (red blood cells become pale in colour) or microcytic (which causes smaller red blood cells), similar to anaemia caused by iron deficiency. In contrast, in cattle and ewes it may be exhibited as hypochromic and microcytic (Suttle *et al*, 1987). Signs of oxidative stress, which is associated with an increase of Heinz body in red blood cells, has also been reported in Cu deficient lambs (Suttle *et al*., 1987).

Copper deficiency can have an adverse effect on immune response through reducing immune function and elevating susceptibility to disease infection (Stable and Spears, 1989). Jones and Suttle (1989) reported that neutrophils from lambs and ewes with plasma Cu below 8 µmol/L (hypocupraemic) had significantly lower *in vitro* killing capacity compared with those with plasma Cu concentration above 8 µmol/L. Woolliams *et al*. (1986a) reported that mortality caused by infection in lambs reared on improved hill pasture were higher in breeds with poor Cu utilisation such as Scottish Blackface compared with Welsh Mountain. Innate immune function as measured by neutrophil phagocytosis (Xin *et al*., 1991) or superoxide dismutase activity (Boyne and Arthur, 1986) and adaptive immune function as measured by lymphocyte proliferation (Arthington *et al*., 1996) and antibody production (Gengelbach and Spears, 1998) have been shown to be reduced in secondary Cu deficiency.

1.4. Copper toxicity

Despite Cu being an essential trace element required for numerous vital functions in the body, it can also be extremely toxic to ruminants (Suttle, 2010). There are marked variations between domestic animals regarding their tolerance to increased dietary Cu intakes (Howell and Gooneratne, 1987). Sheep are considered to be most sensitive to Cu toxicity compared with other species (Ivan, 1993; NRC, 2005). This has been attributed to their inability to increase biliary Cu excretion in response to increased Cu intake (Bremner, 1998) and limited ability to accumulate Cu bound to metallothionein (MT) in their livers (Howell and Gooneratne, 1987). Young animals are more sensitive than adults because they have a higher efficiency of absorption (NRC, 2005). Non-ruminants are more tolerant to Cu toxicity than ruminants and pigs are routinely fed diets with Cu levels of 125-250 mg/kg DM (well above physiological requirement in pigs; 3-4 mg/kg DM; NRC; 1996) to promote growth and feed efficiency (Hill *et al*., 2000). NRC (2005) set the maximum tolerable dietary Cu level in sheep at 15 mg/kg DM, similar to the upper limit (15 mg/kg DM) set by European Commission (EC, 2003).

Todd (1972) reported that chronic toxicity mainly occurs in sheep and cattle and clinical signs in cattle are similar to those characteristics for sheep (NRC, 2005). Copper toxicity has been categorised by Ivan (1993) and MacDowell (2003) into two cases; first, acute toxicity, relatively uncommon, which may arise by accidental increase in Cu intake after either large oral doses of Cu, improperly formulated diets, or following parental injection of Cu in order to treat Cu deficient incidence. During acute Cu toxicity, animals may experience abdominal pain, diarrhoea, and sometimes sudden death. This usually occurs within 3 days following injection in sheep and 12 days in calves (Ivan, 1993). Haemolysis of red blood cells is also found in response to acute Cu toxicity (Linder and Hazegh-Azam, 1996). The second form is chronic Cu toxicity or chronic copper poisoning (CCP) and is found mainly in ruminants rather than mono-gastric animal and only rarely in humans (MacDowell, 2003).

In ruminants CCP occurs in two phases; pre-haemolytic, which is characterised by accumulation of liver Cu over a period of weeks or months until it reaches a level of 1000-1500 mg/kg DM without clinical signs and is given the term ‘silent’, and only when the liver Cu storage is overloaded, hepatocyte damage occurs, resulting in hepatocyte bursting (lysis) (Suttle, 2010; Marta Lopez-Alonso, 2012; Kumaratilake, 2014). The signs that have been characterised as liver necrosis resulting from haemolytic crisis typically exhibit post-mortem as orange livers, with haemolytic anaemia, jaundice, discoloration of digests and tissues with dark kidneys (Bidewell *et al*., 2012). Histological changes such as an increase in the number and size of lysosomes, which is in accordance with increased lysosomal

enzyme activity also been noted in Cu loaded livers (Gooneratne *et al*., 1980). However, the Cu induces haemolysis mechanism is unclear, but may be related to Cu inducing production of superoxide radicals that result in erythrocyte membrane damage (Howell and Gooneratne, 1987).

Some sheep breeds have been shown to be susceptible to Cu toxicity at lower dietary concentrations, especially when dietary Mo and S are below the range (1-2 mg/kg DM and 1.5-2.5 g/kg DM respectively) (MacPherson *et al*., 1997). The tolerable concentration of Cu in the sheep diets is greatly affected by genetics, dietary concentration of Mo and S, period of exposure, dietary source of Cu, and diet composition. Suttle (1977) reported that high mortality has been found as a result of Cu toxicity in Finish Landrace lambs compared with Scottish Blackface, when these lambs were fed a diet containing 45.3 mg/kg DM of Cu. Suttle *et al*. (2002) demonstrated that liver Cu concentrations in Texel lambs fed a ration with moderate Cu concentration (6.1 mg/kg DM) increased to a marginally toxic level, while in some Suffolk and Charollais lambs fed the same diet, liver Cu concentrations reached a marginal toxic level.

Moreover, 89 cases of Cu toxicity were recorded in sheep from diagnostic submission to SAC Consulting Veterinary Service (SAC C VS) from 2011 to 2015 and it is revealed that 51% of 78 cases, that recorded for breed, were in Texel's breed (SAC C VS Surveillance, 2016). Liver Cu concentration of 73 of the 89 cases ranged from 577.3 to 4396.8 mg/kg DM, with a median value of 1548.8 mg/kg DM. Over the past two decades there has been a trend for cattle to be fed increasing amounts of Cu in their diet which has resulted in an increase in the number of cases of Cu toxicity (Livesey *et al*., 2002). Copper was previously viewed as a safe element for cattle. However, cattle now are showing significant accumulation of hepatic Cu at levels that are much lower than cited by ARC (1980). In a recent survey of UK dairy farms intakes of Cu were 120% in excess of NRC (2001) recommended levels (Sinclair and Atkins, 2015), and these high levels may cause Cu toxicity.

1.5. Metabolism of copper in ruminants

Copper metabolism covers the absorption, transportation, storage, and excretion of the element to try to maintain homeostasis (Mercer, 1997). Certain aspects of Cu metabolism must be described in order to fully understand the variable effects of Cu deficiency and toxicity on livestock (Suttle, 2010). Modification to fluctuations in Cu supply is achieved by controlling Cu metabolism (Underwood and Suttle, 1999). There is a wide difference among species of the relative importance of each process and outcome in terms of relationships between dietary Cu supply and liver Cu storage and excretion (Fig. 1.1). Ruminants have generally adapted to a poor Cu supply and have poor control over absorption and excretion but actively store Cu in their livers (Suttle, 2010). By comparison, non-ruminants generally have an adequate supply, have control over absorption and excretion and store only a small quantity (NRC, 2005; Suttle, 2010). However, within ruminants there is genetic influence in Cu haemostasis. For example, Woolliams *et al*., (1982; 1983) described differences in sheep breed with regards to susceptibility to Cu toxicity and deficiency. Similar differences in cattle breeds have also been described (Ward *et al*., 1995; Mullis *et al*., 2003; Fry *et al*., 2013).

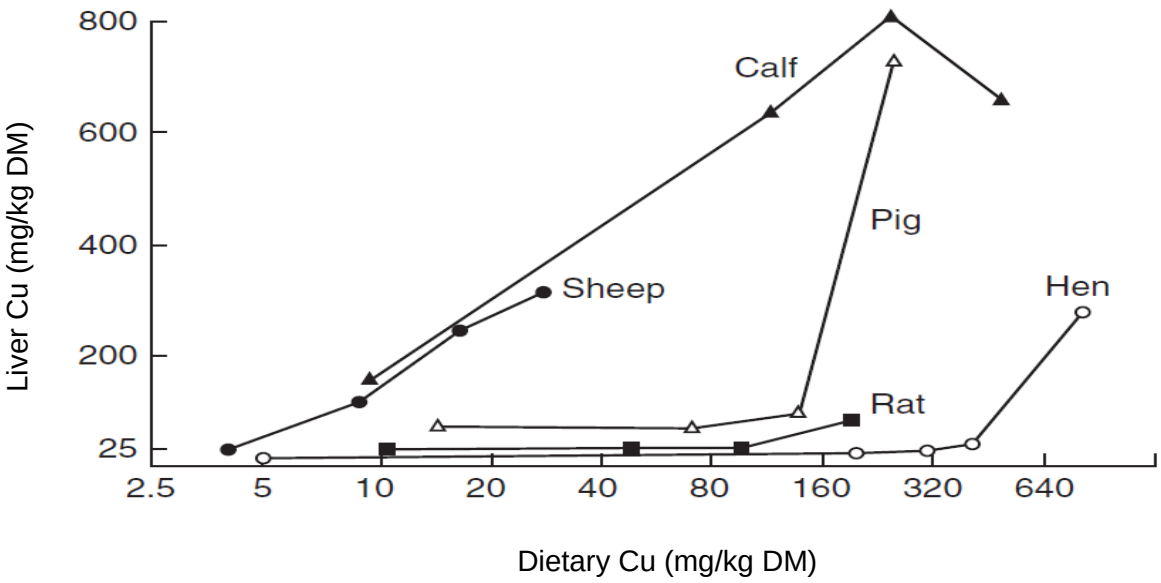


Figure 1.1. Species differ in the extent to which they store excess dietary copper in their livers. Ruminant species, for which the risk of copper deficiency is ever-present, store copper avidly while non-ruminant species, which are rarely at risk, do not.

Source: Suttle (2010)

1.5.1. Absorption

Copper absorption in mammals occurs primarily in the small intestine (Linder and Hazegh-Azam, 1996), and in ruminants it is principally in the duodenum (Cousin, 1985; Kalinowski *et al*., 2016), although the jejunum and ileum may also contribute to Cu absorption (Bremner, 1980). The efficiency of Cu absorption has been found to be partially regulated by the dietary Cu content; when dietary Cu intake increases, the efficiency of Cu absorption decreases and vice versa, in order to maintain homeostasis (Trunland, 1989). There was a linear relationship at low doses up to 12 µg/300 g rat between the amount of oral administrated ^{64}Cu and the rate of entry ^{64}Cu into plasma in rats, which was 30%, whereas at higher doses 12-36 µg/ 300 g rat this relationship became non-linear and the absorption rate reduced to 13% (Merceau *et al*., 1970). Studies have indicated that the mechanism of Cu uptake across brush border microvilli can vary depending on the Cu concentration in the intestinal cells. Two different mechanisms have been proposed for Cu absorption from the mucosal to serosal side of the gastrointestinal tract; first, at low Cu concentrations, absorption was found to be mediated through non-energy dependent saturable carrier (active transport) and second, at higher concentrations it was found to occur via diffusion (Linder and Hazegh-Azam, 1996; Kalinowski *et al*., 2016).

Studies with laboratory animals have indicated that Cu transporter protein 1 (Ctr1) is the principal protein that is responsible for Cu uptake across the microvilli (Harris, 2000; Lee *et al*., 2002; Suttle,2010) along with other transports such as divalent metal transporter 1 (DMT1), and Cu transporter 2 (Ctr2) (Arrendodo *et al*., 2003; Blair *et al*., 2009; Suttle, 2010). Recent studies suggest that Ctr1 is an integral membrane protein that is primarily responsible for importing dietary Cu across the brush border (Fig. 1.2), forming a homotrimeric pore that is specific to Cu in the cuprous state Cu^{+1}. However, dietary Cu is present in the cupric form Cu^{+2} and it needs to be reduced before uptake. Therefore, Cu^{+2} is reduced to Cu^{+1} to facilitate absorption (Kalinowski *et al*., 2016), via metalloreductases, or potentially by Fe^{+3} reductance enzymes such as duodenal cytochrome b (Dcytb), which is also able to reduce Cu^{+2} (Knuston, 2007).

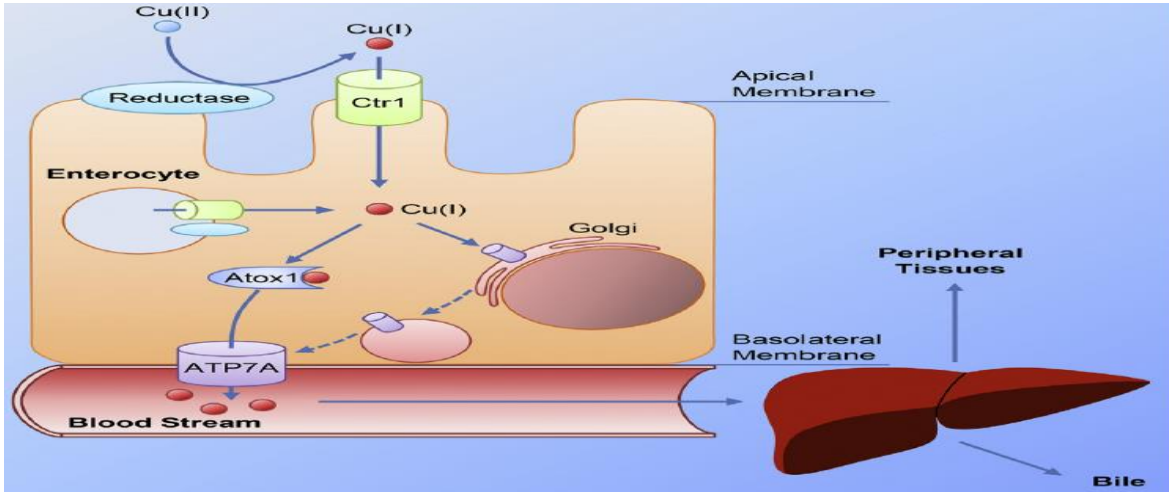


Figure 1.2. Copper absorption across the brush border with the help of copper transporter 1 (Ctr1). Atox1 then shuttles the copper to be pumped out to the blood stream by ATPA. Kalinowski *et al*. (2016)

The imported Cu is delivered by the chaperones COX1, COX17, Atox1, and CCS to the secretory compartment (Lee and Thiele, 2002). Copper is then loaded onto Cu-dependent enzymes, or moved out from the basolateral membrane via a P-type ATPase, ATP7A (Vonk *et al*., 2008), into the portal blood stream, where the Cu binds to albumin or histidine (Suttle, 2010).

It is generally agreed that metallothionein (MT) in intestines regulates Cu absorption, however, it is not clear that MT comprises an essential component of Cu absorption, but it may help protect against Cu toxicity (DiSilvestro and Cousins, 1983). Sheep appear to have a low ability to synthesise MT (Saylor *et al*., 1980). Metallothionein synthesis in enterocytes has also been identified as having a role in the regulation of the Cu absorption (McDowell, 1992), whereby in mucosal cells, absorbed Cu from the intestine binds to MT and is then sloughed off from mucosa and excreted in the faeces (Hartmann *et al*., 1993).

1.5.2. Transportation and intracellular uptake

Once Cu is absorbed from the enterocyte, it is transported in the portal circulation where it is delivered to the liver and other tissues mainly bound to the carrier proteins such as albumin and to a lesser extent transcuprein, although amino acids such as histidine, threonine, and glutamine may also be involved (Linder *et al*., 1998; Valko *et al*., 2005; Vonk *et al*., 2008). The liver is the organ that distributes most of the absorbed Cu and has a central role for regulating Cu homeostasis within hepatocytes (Vonk *et al*., 2008). After Cu uptake by the hepatocyte via Ctr1 (Tapiero *et al*., 2003), Cu is then rapidly incorporated into a variety of intracellular Cu transporters and chaperones (Vonk *et al*., 2008). These chaperones shuttle Cu directly to their specific Cu-dependent proteins or enzymes (Markossian and Kurganov, 2003).

Intracellular Cu transport is performed by chaperones, including Atox1, Cox17 and CCS as illustrated in Fig. 1.3. (Lutsenko *et al*., 2007). Atox1 traffics Cu to the Trans Golgi Network, where Cu is incorporated into Cu proteins such as Cp via ATP7B (Linder, 2010). CCS delivers Cu to Cu/Zn SOD (Prohaska, 2008) and COX17 directs Cu to the mitochondria for incorporation into cytochrome *c* oxidase (Leary *et al*., 2004). Intracellular Cu is bound to MTs for intracellular metal detoxification, which store excess Cu (Wang and Guo, 2006) or is stored in vesicular Cu pools. Data also suggests a potential role for the low-affinity Cu transporter CTR2 in releasing the Cu from these pools (Blair *et al*., 2009). If there is an excess of Cu, ATP7B translocates toward the canalicular membrane, thereby promoting Cu excretion into the bile canaliculus and eventually into the faeces. The interaction of COMMD1 with ATP7B suggests that COMMD1 collaborates with the function of ATP7B. It is suggested to have a role in the ATP7B-mediated vesicular Cu sequestration pathway (La Fontaine *et al*., 2007).

The necessity of intracellular Cu transportation is emphasised by several well-known genetic diseases, such as Menkes disease (MD) and Wilson's disease (WD). The MD is exhibited when ATP7A is mutated, while WD occurs due to ATP7B mutation (La Fountaine *et al*., 2010). ATP7A and B have similar structure and functions, although the clinical exhibition and pathology related to MD are completely different to WD (Kodma *et al*., 2012).

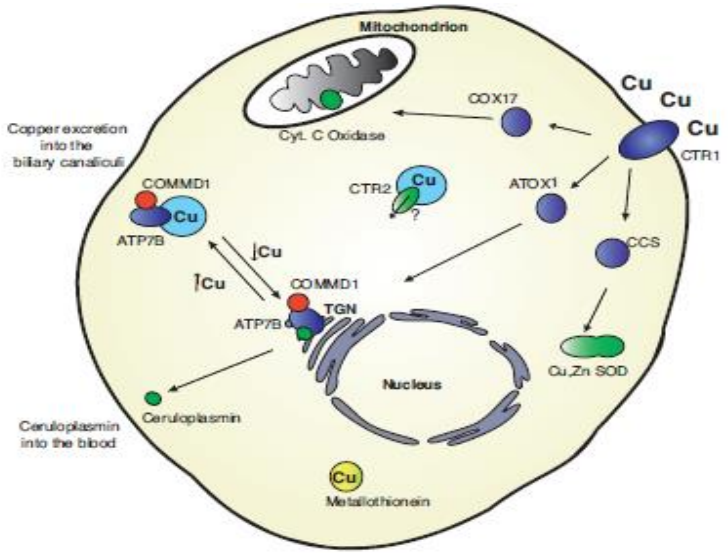


Figure 1.3. Illustrating the intracellular copper pathways in hepatocyte
Vonk *et al*. (2008)

The ATP7A protein is expressed in the majority of tissues except liver. In the animal model of MD, ATP7A is expressed in astrocytes, cerebrocytes, and neurons. This confirms the role of ATP7A in intracellular Cu transportation (Qian *et al*., 1998). ATP7A is a protein that is required for Cu uptake in the small intestine into the blood and also for Cu transport across the blood-brain epithelium (La Fontaine and Mercer, 2007; Lutsenko *et al*., 2007). In addition, ATP7A in transgolgi network (TGN) also pumps Cu to Cu containing enzyme (lysyl oxidase) (La Fontaine and Mercer, 2007). ATP7A protein is coded on the X-chromosome that is missing in human MD due to mutation (Prohaska, 2008). Patients with MD are characterised by low Cu absorption from small intestine and transfer into the blood, resulting in severe Cu deficiency in cells in most organs.

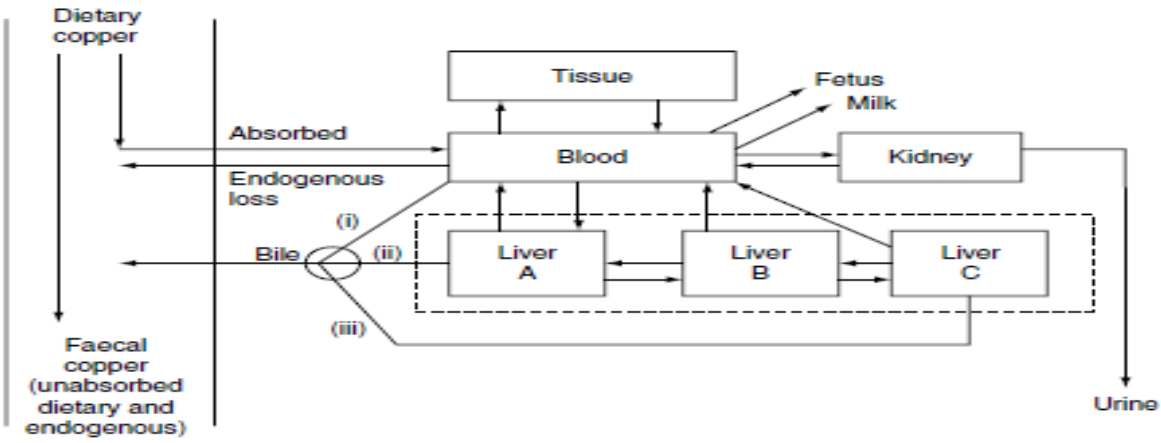
ATP7B is the protein that has much more restricted expression (La Fontaine *et al*., 2010), with the highest expression in the liver (Kodama *et al*, .2012; Wada *et al*., 2014). The role of ATP7B centres on delivering Cu from the cytoplasm of the hepatocyte to the Cp located in the TGN (Lonnerdal, 1996). In addition, ATP7B can excrete Cu from the liver to the bile when the concentration of hepatic becomes elevated (Kodama *et al*., 2012). Patients with WD carry a mutation in the ATP7B gene (Llanos and Mercer, 2002; Shim and Harris, 2003), which results in an accumulation of Cu in the liver, with subsequent deposition of Cu in the central nervous system due to having inactive ATP7B, which cannot excrete liver Cu into the bile or incorporate it into Cp (Fatemi and Sarkar, 2002).

Ceruloplasmin is synthesed in the liver (Goodman *et al*., 2004). It has a half-life of approximately 2-3 days in blood plasma (Linder, 1991). Ceruloplasmin mainly transports Cu from liver to other tissues (Turnlund *et al*., 1998; Vonk *et al*., 2008) and approximately 90-95% of circulation blood Cu is in the form of Cp in animals (Terada *et al*., 1995), while the remaining is loosely bound to albumin and other amino acids (Kodama *et al*., 2012).

1.5.3. Copper storage

After being absorbed from the intestine and transported into the blood Cu is then rapidly stored in the liver (Suttle, 2010; Gaetke *et al*., 2014). The liver is regarded as a main organ for Cu storage (Bremner, 1998) in sheep and it has been found that approximately half of the total Cu in the carcass is stored in the liver (Langlands, *et al*., 1984). In ruminant animals, normal liver Cu concentration ranges from 100-500 mg/kg DM, this level may increase to 2000-3000 mg/kg DM during periods of Cu toxicity (Dick, 1954).

McDonald *et al*. (2011) described a new framework model of the possible Cu movement and storage in the ruminant body based on kinetic models that were proposed by Gooneratne *et al*. (1989b), using ^{64}Cu and ^{67}Cu isotopes respectively in sheep (Figure 1.4). The first pool, box A, represents a temporary liver Cu storage destined for exchange with blood and excretion into bile. The second pool, box B represents a temporary liver Cu storage for incorporation into Cp. The third pool, box C represents a long-term liver Cu storage compartment from which excretion into bile and secretion into blood.



(McDonald *et al*., 2011)

Figure 1.4. The possible copper movement and storage within the body within the body.

In mammals, a series of metabolic processes are carried out within liver Cu, preparing Cu ions for subsequent incorporation into proteins for storage, transport and excretion (Gaetke

et al., 2014). Bremner (1998) and Gaetke *et al*. (2014) proposed three distinct pathways involved in this process; first, preparation of Cu for secretion into the bile. Second, temporary storage of Cu in the liver by binding with MT. Third, the incorporation of Cu into the Cp for distribution throughout the body.

The sub distribution of Cu in hepatocytes is important in order to elucidate changes that occur during Cu toxicity (Bremner, 1998). In normal circumstances, the distribution of Cu in the hepatocyte is 20% in the nuclear portion, 10% in microsomes, and 20% in the large granules of mitochondria and lysosomes, and the remainder is deposited in the cytosol either in Cu-depending enzymes or MT (Saylor *et al*., 1980; Bremner, 1998). The distribution of Cu in the subcellular fraction of the hepatocyte depends on total liver Cu rather than the physiological status of the animal, such as age, species or Cu status (Kumaratilake, 2014). When reaching a certain threshold of Cu concentration within cells, any Cu excess is sequestered into the lysosomes as a part of detoxification process where Cu ions are not available to initiate toxic effect (Kumaratilake, 2014). The kidneys are regarded as the second site of Cu storage, after the liver becomes saturated (Walsh, 1968).

1.5.4. Copper excretion

Biliary Cu secretion is quiescent in the foetus, while it is initiated after birth (Prohaska, 2006) in order to allow both the entero-hepatic recycling of Cu and the excretion of overload Cu. In sheep, biliary Cu excretion can increase as liver Cu concentrations rise (Grase *et al*., 1998) however breeds may vary in this respect (Suttle *et al*., 2002). At high Cu intakes, the alleviation in hepatic Cu storage in cattle was quicker than sheep by means of biliary excretion (Phillippo and Graca, 1983). The lower threshold for Cu storage in lysosomes in the bovine may explain this difference (Lopez-Alonso *et al*., 2005). Compared with other mammals, sheep have a variant Cu phenotype and do not efficiently excrete Cu via the bile. Goats retained 6 to 9 times less Cu in their liver compared with sheep when exposed to high Cu intakes (30-60 mg/kg DM) (Zervas *et al*., 1990) and may also have a well-developed capacity for biliary Cu secretion. In sheep, the excretion of Cu from liver into the bile can be indirect via sequestering excess Cu as a response to Cu loading from Cu loading hepatocytes which elevate the number and size of lysosomes, paralleled by a rise in lysosomal enzyme activity (Gooneratne *et al*., 1980; Kumaratilake, 2014). There is also a direct path by which Cu is removed from hepatocyte lysosomes into the bile after intravenous or intraduodenal administration of tetrathiomolybdate (Gooneratne *et al*., 1989b; Gooneratne, 2012).

Gooneratne and Christensen (1997) claimed that dietary Cu increase in sheep does not necessarily cause a large increase in biliary Cu excretion, even a reduction in biliary Cu

excretion has been reported by increasing dietary Cu offered to Romney-Marsh lambs from 6.78 to 17.78 mg/d (Grace and Gooden, 1980). Moreover, Gooneratne (2012) confirmed that increasing dietary Cu in sheep from 6.3 to 41.6 mg/kg DM did not elevate biliary Cu excretion. However, increasing Cu herbage concentrations from 8.1 to 40.5 mg/kg DM, elevated sheep bile Cu concentration from 0.76 to 1.97 mg/kg bile (Grace *et al*., 1998). Urinary excretion of Cu is normally small (less than 1% of ingested Cu) (Grace and Gooden, 1980; Buckely, 1991), constant and significantly unaffected by Cu intake in all species, although it is increased by exposure to Mo (Gooneratne *et al*., 1981) in sheep, and (Gooneratne *et al*., 1987) in cattle. The majority of the endogenous losses of Cu come from biliary excretion and the rest are derived from saliva, gastric, intestinal juices, and Cu in desquamated mucosal cells (Gooneratne *et al*., 1981; Kim *et al*., 2008). High dietary Cu concentration in lambs increased faecal Cu concentration (Zevras *et al*., 1990).

1.6. Copper metabolic interactions

1.6.1. Copper sulfur interactions

Sulfur is found in all animal feed types and ranges in pasture from 0.5 to more than 5 g/kg DM (Suttle, 2010). Sulfur is present in plants as a component of the S containing amino acids methionine and cysteine. However, it can also be present as inorganic sulfate (MacPherson, 2000). Ruminant animals are unique due to the ability of ruminal micro-organisms to use the sulfate to produce S-containing amino acids and the B vitamins, thiamine and biotin (Suttle, 2010; McDonald *et al*., 2011). Within the rumen, sulfate-reducing bacteria produce sulfide from dietary sulfur (as inorganic sulfur or sulfur amino acids) (Spears, 2003; Drewnoski *et al*., 2014). Sulfide production in the rumen is dependent on the ruminal degradability of dietary protein. This in turn depends on both the protein level of the diet and protein solubility (Ivan, 1993). In sheep, increasing dietary S intake from 0.6 to 1.9 to 3.4 g/d as organic cystine or inorganic SO_4 forms resulted in an increased ruminal sulfide concentration (Bird, 1970).

Rumen hydrogen sulfide can be either absorbed by the rumen or inhaled via the lungs (Kandylis, 1984). Inhalation of sulfide from the rumen of cattle and sheep consuming feed (Gould *et al*., 1991; Hill and Ebbett, 1997) or water (Wagner *et al*., 1998) high in sulfate has been implicated as a potential cause of respiratory problems or polioencephalomalacia (PEM) in ruminants. Polioencephalomalacia, which is a neurological disorder and can be fatal, is possibly induced by the inhalation of hydrogen sulfide produced in the rumen (Gould, 1998). In cattle, the incidence of PEM was found to be small (0.14%). When diets containing 4.6 g S/kg DM and when dietary S levels were between 4.7-5.6 g/kg DM, the incidences of PEM increased to 0.35%. By increasing the dietary S level to above 5.6 g/kg DM, the incidence of PEM was 6.06% (Vanness *et al*., 2009). Sulfur if consumed in excess, has been shown to reduce Cu availability and animal performance through the formation of unabsorbable Cu-sulfide compound (Spears, 2003; Suttle, 2010). Bird (1970) reported that omasal flow of soluble Cu was reduced by approximately 50% in sheep, when dietary S increased from 0.8 to 2.5 g/kg DM. Suttle (1974) demonstrated that increasing dietary S concentration as organic (methionine) or inorganic sulfate from 1 to 4 g/kg DM in hypocupraimic ewes fed low molybdenum diets (0.5 mg/kg DM of Mo), markedly reduced lambs Cu availability based on a plasma Cu response. Moreover, applying ammonium sulfate fertiliser (67 kg/ha) to Bahiagrass in order to increase S level in S deficient plants resulted in substantially increased S level compared to unfertilised pastures (5.1 and 2.5 g/kg respectively). This in turn caused a dramatic reduction in the liver Cu concentration of cattle grazing on ammonium sulfate fertilised pasture that had a lower liver Cu concentration (72 mg/kg DM) compared with those grazed on unfertilised pasture (204 mg/kg DM) (Arthington *et al*., 2002).

1.6.2. Copper-molybdenum-sulfur interactions

Molybdenum levels in forages are reported to be relatively low (1-2 mg/kg DM), although in certain areas of the UK Mo can be as high as 10 mg/kg DM (Suttle, 2008a). In early investigations, biological antagonism between Cu and Mo was observed when Red Devon cattle grazing in ‘’teart’’ pasture of Somerset rich in Mo were found to exhibit clinical Cu deficiency exhibiting signs such as scouring and a change in coat colour (Ferguson *et al*., 1943). However, the antagonist effects of Mo on Cu availability are suggested to be in part dependent on the dietary S content (Dick, 1953; Dick *et al*., 1975; Spears, 2003). It has been shown that increasing dietary Mo intake to 10 mg/day decreased liver Cu storage over a period of 3 months when lucerne hay was offered to sheep, although, the same increase in Mo intake had no effect on liver Cu storage in sheep fed on an oaten hay diet (Dick, 1953). However, when inorganic sulfate (potassium sulfate) was added to oaten hay so that sheep on the two diets had the same intake of sulfate, as well as Cu and Mo, the difference between the diets in the effect of Mo on liver Cu storage was eliminated (Dick, 1953). Similarly, in sheep, Cu bioavailability was found not to be affected when dietary Mo increased from 0.5 to 4.5 mg/kg DM and S content was only 1 g/kg DM, whereas Cu availability was reduced by 40-70% by the addition of 3 g S/kg DM and 4 mg Mo/kg DM to the diet containing 1 g/kg DM and 0.5 mg/kg DM of S and Mo, respectively (Suttle, 1975).

The biological interactions between Cu, Mo, and S has been extensively studied and reviewed Mason (1982), Gould and Kendall (2011), and Sinclair and Mackenzie (2013). Dietary Mo is absorbed readily and rapidly (Mills and Davis, 1987). However, in the presence of the rumen sulfide, which is formed from reduced dietary S such as inorganic sources of sulfur or sulfur-containing amino acids by micro-organisms in the rumen (Chidambaram *et al*., 1984), Mo can combine with sulfide, producing thiomolybdate compounds that have a high affinity to complex with Cu and form biologically unavailable Cu-thiomolybdate complexes (Dick *et al*., 1975). Suttle and McLauchlan (1976) stated that in the presence of dietary Mo and S, true Cu availability can be estimated in sheep using the following equation:

$$\text{Log } A = - 1.153 - 0.076 \text{ (S)} - 0.013 \text{ (S x Mo)}$$

Where (*A*) is true Cu availability (mg/mg), S (g/kg DM sulfur), and Mo (mg/kg DM molybdenum). However, this equation does not take into account the effect of dietary Fe on Cu absorption (Symonds and Forbes, 1993).

The mechanism by which thiomolybdates are produced in the rumen is proposed to be a stepwise hydrolysis of molybdate (MoO_4) with an oxygen (O) (from a water molecule (H_2O)) being substituted by an S from a sulfide (H_2S) donor at each step (Gould and Kendall, 2011). This reaction is reversible and equilibria is dependent on pH and temperature (Gould and Kendall, 2011). Clarke and Lurie (1980) demonstrated the formation of the relative thiomolybdate, $MoO_nS_{4-n}^{2-}$ from molybdate and sulphide salts in aqueous media under a condition simulate the anaerobic rumen environment by a series of substitution between O^{2-} and S^{2-} (as presented below);

$MoO_4^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_3S^{2-}$	Monothiomolybdate
$MoO_3S^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_2S_2^{2-}$	Dithiomolybdate
$MoO_2S_2^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoOS_3^{2-}$	Trithiomolybdate
$MoOS_3^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoS_4^{2-}$	Tetrathiomolybdate

Source: Clarke and Lurie (1980) and Gould and Kendall (2011)

Tri and tetra-TMs have been suggested to be responsible for inhibition of Cu absorption within the rumen, while post-absorptive effects on Cu metabolism are possibily related to di and tri-TM (Price *et al*., 1987). Later, Osman (1988) suggested that tetra-TM also may be involved in post-absorptive effects on Cu metabolism such as inhibition of enzyme activity. The TMs have been suggested to be unstable in acid solution, however, when they associated with the solid phase of rumen digesta they are more stable (Gould and Kendall, 2011). In contrast, unbounded thiomolybdates in the liquid phase are readily hydrolysed in the abomasum. Copper is usually associated with the solid phase in the rumen digesta (Allen and Gawthorne, 1987), therefore possibly facilitating intraruminal formation of Cu-TM (Gould and Kendall, 2011).

Ultimately, at low Cu:Mo ratio (<1:1) or high Mo exposure (> 8 mg/kg DM) in sheep, the excess of rumen TM may leave rumen, when Cu is not available for interaction, and transports into the bloodstream (Price *et al*., 1987), causing a systemic effect mainly involving the inhibition of Cu metabolism (Suttle, 1991). The relative importance of the systemic effect of absorbed thiomolybdates on blood Cu compartment appears to increase after observing a temporary elevation in plasma Cu concentration (Kumaratilake and Howell, 1989; Suttle, 2008a; Gooneratne, 2012). Hynes *et al*. (1984) demonstrated that circulating TMs in the blood led to a decrease TCA sCu fraction of plasma Cu and increased in TCA-insoluble Cu, which is identified as Cu bound to albumin. Suttle (1991) proposed a two fold physiological effects of Cu-TM-albumin in the bloodstream; the first, to trap the availability of Cu absorbed from the gut and delivered to the liver through the hepatic portal

vein, for synthesis of Cp. The second, to limit the available TM in an effective detoxification mechanism.

Parental dose of tetra-TM in sheep has been shown to inactivate SOD activity (Suttle *et al*., 1992). Similarly, it has been shown that tetra-TM *in vitro* inhibits functions of a wide range of Cu enzymes such as Cp, cytochrome oxidase, SOD activity, ascorbate oxidase, tyrosinase (Chidambaram *et al*., 1984). However, Suttle (2008a) reported that the addition of tetra-TM (therapeutic dose) did not inhibit Cp activity in sheep, which is possibly related to the affinity of TM to bind to the albumin rather than Cp (Hynes *et al*., 1984). Moreover, TM has been used as a treatment for CCP in sheep (Humphries *et al*., 1986; Haywood *et al*., 2004) due to promoting rapid clearance of the body Cu via biliary-faecal route (Manson *et al*., 1988). Gooneratne (2012) reported that administration of TTM (IV) to sheep fed a diet with high dietary Cu (41.6 mg/kg DM) resulted in increased Cu bile excretion due to removing Cu from hepatocytes as evidenced by a rise in lysosomal activity (β-glucuroindase).

1.6.3. Copper-iron interactions

Ruminants grazing on pasture or consuming forage-based diets are often exposed to excessive levels of Fe (> 500 mg/kg DM; NRC, 2005) due to soil contamination of forages (Standish *et al*., 1971), soil ingestion (Suttle and Peter, 1985), or high Fe levels in some feedstuffs such as alfalfa, soyhull, and grass silage (NRC, 1996; DePeters *et al*., 2000). Between farms, the herbage Fe level can vary greatly, ranging from 150-1345 mg/kg DM Fe (Nicol *et al*., 2003). High Fe levels are often reported in overgrazed pasture and in soils prone to waterlogging (81-2300 mg/kg DM Fe), or ground water soil (200-1000 mg/kg DM Fe). In both Britain and New Zealand, pasture contamination by soil has been related to increased Fe intake by ruminants (Suttle *et al*., 1975). This has been confirmed by Healy *et al*. (1972) who reported that soil ingestion in out wintered sheep and cattle can constitute 10-25% of the total DM intake. Vaithiyanathan and Singh (1994) also reported that daily soil intake in sheep reared in arid areas can be 163 g/d.

Suttle *et al*. (1982) reported that Cu absorption was dramatically decreased in ewes supplemented with either a chalky or clay soil, containing 2400 or 1400 mg/kg DM Fe. Therefore, the contamination of herbage and silage by soil or soil ingestion should be taken into consideration as a considerable proportion of soil in the digestive tract could become soluble (Healy, 1972). Hansen and Spears (2009) showed that Fe bioavailability from soil contamination of harvested maize green chop was elevated following *in vitro* ensiling. In addition to a Cu-Mo-S complex reducing Cu availability, the exposure of sheep and cattle to high levels of Fe (500-800 mg/kg DM) (Table 1.4) have been shown to result in a dramatic reduction in liver Cu concentration, especially in cattle, to levels which indicated severe Cu deficiency. Clinical signs were not observed such as reduced growth and change in hair texture or colour (Phillippo *et al*., 1987a; 1987b). However, additional Mo (5 mg/kg DM) produced clinical signs in cattle and calves (Phillippo *et al*., 1987a; 1987b). Therefore, clinical signs of Cu deficiency could be more related to Mo rather than Fe antagonists.

Table 1.4 The effect of additional dietary iron (mg/kg DM) on liver copper concentration (mg/kg DM) in experimental animals.

References	Animal type	Additional Fe	Duration (week)	liver Cu at week 0	Final liver Cu (additional Fe)
Phillippo *et al*.(1987a)	calves	800	32	94.5	3.6
Phillippo *et al*.(1987b)	cattle	500	32	134	5.5
Williams (2004)	lamb	500	10	278	~90
Sefdeen *et al*. (2014)	lamb	750	6	313	205
Sefdeen *et al*. (2016)	lamb	800	13	545	274

Liver Cu concentration (mg/kg DM).

The mechanisms by which Fe reduces Cu absorption has been proposed as; first, Fe in the rumen interacts with sulfide and Cu, producing an insoluble Fe-Cu-S complex, alternatively, Fe combines with sulfide producing Fe-S and then Cu exchanges with Fe to form insoluble Cu-S complex (Gould and Kendall, 2011). The second, down regulation of the non-specific carrier DMT1 via soluble Fe, leading to prevent the Cu from binding to DMT1 due to competition (Garrick *et al*., 2003). The role of DMT1 in intestinal Fe^{+2} absorption is well known (Arredondo *et al*., 2003; Kalinowski *et al*., 2016), and a recent experiment has indicated that DMT1 is physiologically related to Cu^{+1} carrier in intestinal cell, the presence of DMT1 in duodenum has been found in cattle (Hansen and Spears, 2008). Concentrations of intestinal DMT1 are regulated by Fe status in the body as well as dietary Fe concentrations. Thus, high dietary Fe may lead to impaired absorption of Cu (Hansen et al., 2008).

1.6.4. Copper zinc interactions

Zinc requirement for sheep has been set at 20-33 mg/kg DM (NRC, 1985). The level of zinc in pasture, according to the values recorded worldwide, can be lie between 7-100 mg/kg DM, with an average value falling between 20 and 36 mg/kg DM (Minson, 1990; NRC, 2005). Industrial pollution can elevate Zn concentration in grass up to 5 to 50 fold (Mills and Dalgarno, 1972). The level of dietary Zn required to have a significant effect on Cu absorption must be at least 20 times higher than the recommended level (NRC, 2001). Bremner *et al*. (1976) demonstrated that increasing the dietary Zn level from 43 to 220 or 420 mg/kg DM effectively reduced liver Cu concentration and liver damage, and prevented the onset of a haemolysis crisis in lambs' feeding on a diet containing high level of Cu (29 mg/kg DM).

However, these levels of Zn are unlikely to occur across wide range of forages, but are possibly elicited during the treatment of facial eczema via large dose of Zn as zinc salt (Suttle, 2010). The protective effect of high Zn intake against facial eczema disease in sheep and cattle in New Zealand is well recognised and characterised by liver damage, loss of weight, photosensitising lesions particularly on the face, and death (Suttle, 2010). Van der Schee (1983) showed that increasing the dietary Zn concentration in Texel lambs, fed a diet containing 34 mg/kg DM Cu, from 45 to 225 and 479 mg/kg DM resulted in decreased liver Cu concentration from 1652 to 1310 and 1158 mg/kg DM respectively. Likewise, Smith *et al*. (2010) reported that supplementing Zinc oxide (ZnO) as a bolus releasing 6.628 g Zn/day to prevent facial eczema in dairy cows, which was also supplemented with 150 mg Cu/day, substantially reduced liver Cu concentration by 50%. The mechanism underlying the interaction between Zn and Cu has been proposed to potentially involve MT as Zn is suggested to induce MT (Tacnet *et al*., 1991). It has been suggested that Zn displaced Cu from the sulfhydryl binding site on MT and hence Cu bound to MT is excreted into the intestinal lumen (Gooneratne *et al*., 1989a).

1.7. Effect of forage type on copper metabolism

The importance of the effect of forage type on Cu availability centers around the ability of the diet to meet Cu requirement and depends more on Cu availability rather than Cu concentration in the diet (Suttle, 2010). The aetiology of Cu deficiency in grazed ruminants has been partially associated with low Cu availability in fresh forages compared with conserved (Suttle, 1986). In early work on Cu deficiency related symptoms (diarrhoea) in cattle grazing on forages containing a high Mo concentration, clinical signs ceased when the herbage was fed as hay (Ferguson *et al*., 1943). Allaway (1977) also reported that diarrhoea in cattle grazing in fresh forages disappeared when the same forage was dried as hay. These authors suggested that drying forages improves Cu availability possibly due to a decrease in the availability of the water-soluble molybdate content in forages when preserved as hay (NRC, 1985).

The effect of preservation method on Cu metabolism has been confirmed by Fishers *et al*. (1972) who reported that plasma Cu concentration was found to be higher in cows fed hay compared with grass silage. Similarly, the coefficient of Cu absorption in sheep has been found to be higher in hay and dried grass compared with fresh grass and grass silage (Table 1.6). It appears that Mo and S were similar across forages, except in fresh grass, which had a higher Mo content. In addition, in silage the Cu concentration was approximately 3 times as much as other forages, while it had the lowest Cu absorption. It is suggested that preserving grass as silage rather than hay may result in a decrease in Cu status in ruminants (Suttle, 1980b).

Table 1.6 illustrates the absorbability of copper (%) in fresh and conserved grass fed to sheep using repletion technique.

Forages	Sample no.	Copper mg/kg DM	Molybdenum mg/kg DM	Sulfur g/kg DM	True absorption of copper (%)
Fresh grass	4	7.5	3.0	3.2	2.4
Silage	3	18.8	1.0	3.6	1.3
Dried grass	2	6.6	0.9	3.6	4.0
Hay	3	8.1	1.2	3.4	6.1

Adapted from (Suttle, 1980b).

The reasons for the effect of preservation method on Cu availability is not clear (Suttle, 1983a), but could be related to the difference in dietary protein degradability in the rumen (Ivan, 1993). It has been suggested that the higher degradability of dietary protein from fresh grass may contribute to an increase a rumen sulfide, which in turn, reduces Cu availability via the formation of insoluble Cu-S complexes (Ivan, 1993). Similarly, it has been reported that animals grazed on summer pasture had higher plasma Cu concentration compared with animals grazing on lush pasture, possibly due to the lower protein degradability in dry pasture (Gawthorne, 1987). In addition, highly fermentable carbohydrate feedstuffs such as cereals could reduce rumen pH, which in turn, may increase sulfide absorption or break down rumen thiomolybdates, consequently enhancing Cu availability (Suttle, 1991). Further evidence of the effect of low rumen pH from ruminants being offered a silage diet in reducing potency of Cu-Mo-S interaction comes from Wang *et al*. (1988) who reported that clinical signs of Cu deficiency (diarrhoea) were exhibited in steers offered grass silage, containing 35 mg of Mo/kg DM, over a period of 13-14 weeks, while similar Mo concentration in pasture induced immediate diarrhoea (Ferguson *et al*., 1943). Moreover, the coefficient of Cu absorption has been shown to be higher in feedstuffs low in fibre such as cereals (0.091) compared with feedstuffs high in fibre such as fresh herbage (0.025), silage (0.049), and hay (0.073) (Suttle, 1986; Suttle, 2010). The effect of fibre content on reducing Cu absorption has been suggested to be possibly due to irreversibly binding with Cu, or due to indirectly elevating the dwell time in the rumen, the site of CuxMoxS interactions (Suttle, 1991).

The inhibitory effect of antagonists on Cu metabolism has been reported to be affected by the basal diet being fed to animals, with Suttle (1983b) demonstrated that the inhibitory effect of additional Mo on Cu absorption in sheep was less in grass hay compared with semi-purified diet or fresh herbage, suggesting that preservation forage could decrease the Cu-Mo-S antagonism possibly due to lower release of Cu from dried feed into the rumen, the site of CuxMoxS interaction (Suttle, 1983b; 1986). The lower release of Cu from grass hay in the study by Plane *et al*. (1978) compared with grass silage (Rooke *et al*., 1983) during rumen *in sacco* incubation has been related to the higher lignified cell wall of the grass hay compared to grass silage, which may have sequestered a portion of Cu and prevented its release into the rumen. This is further confirmed by the work of Ibrahim *et al*. (1990) which showed that Cu solubility in maize silage in acid detergent solution (ADS), which provides information on the proportion of minerals associated with the plant cell, was greater compared with rice straw at 95% and 66%, respectively.

Furthermore, Hart *et al*. (2011) reported that liver Cu concentration was not affected by the addition of Mo and S in the cows offered maize silage (Hart *et al*., 2011), whereas Sinclair *et al*. (2013) demonstrated that additional Mo and S in dairy cows fed grass silage markedly reduced liver Cu concentration. Recently, Sinclair *et al*. (2017) reported that liver Cu status was higher in cows fed maize silage compared with grass silage and additional Mo and S resulted in a reduction in liver Cu status with the greater extent in the grass silage fed cows than the maize silage fed cows. However, the reason for this effect was not clear. Therefore, it appears that the antagonist effect of additional Mo and S on Cu metabolism may be infuenced by forage type and preservation method, although the mechanism of this effect is not clear (Suttle, 2010), it may be pH dependent.

1.8. Conclusion

Ruminant animals are vulnerable to copper (Cu) deficiency because of rumen sulfide generation which then binds with molybdate and lowers copper availability from forages (Suttle, 2012). Copper deficiency around the UK has been found as a problem which can lead to economic and production losses such as an impairment of growth, infertility, alteration hair or wool (Suttle, 2010). Copper deficiency in ruminants is attributed to consumption of diets or forages that have insufficient copper (primary), or when the overall level of Cu in the diet seems sufficient but not available for biological functions due to intraruminal reactions that occur between Mo and S, producing thiomolybdate compounds which have a high affinity to interfere with Cu and reduce Cu absorption. While, in the absence of rumen-available Cu, thiomolybdates are able to be absorbed by rumen wall into the blood stream and deactivate Cu-containing enzymes and proteins, producing clinical disturbances (Suttle, 1991; Suttle, 2010; Gould and Kendall, 2011). Sinclair *et al*. (2017) investigated the how role of forage type affects the copper status in dairy cows. It was found that the effects of supplemental Mo and S were influenced by forage type. The effect on the liver Cu status was more pronounced in the case of the grass silage than the case of the maize silage diet supplemented with the antagonists. This would imply that dietary Cu was more available for uptake by the animal, or that interactions between antagonists and copper were more pronounced in the grass based diet. There has been limited studies on the effects of different forage types and how this impacts copper mobilisation in ruminants. Therefore, this thesis will attempt to further understanding of copper metabolism in ruminants by analysing the effects of the forage type on rumen fermentation characteristics and determining their effects on copper antagonist interaction between forage type and mineral levels in growing lambs. Additionally, it will investigate distribution of minerals between solid and liquid phases of the digesta in the rumen of animals fed different forages and how it’s affected by the inclusion of Mo and S.

Chapter 2. General materials and methods

2.1. Forage and concentrate analysis

Feed samples from all experimental Chapters were analysed at Harper Adams University according to the method of AOAC (2012) for DM (934.01), CP (990.03), EE (2003.5), and ash (942.05). NDF was analysed according to Van Soest *et al*. (1991) with the use of a heat-stable α-amylase (Sigma, Gillingham, UK), and expressed exclusive of residual ash. All feed samples were analysed in duplicate.

2.1.1. Dry matter

The dry matter (DM) content of feed samples was determined after weighing approximately 100 g into a pre-weighed aluminium tray. Samples were then placed into a force-draught oven (Binder, Tuttlingen, Germany) at 105 °C overnight, and dried until constant weight. All subsequent laboratory analysis was carried out on dried samples ground through a 1 mm screen or a 3 mm 3creen (for the *in vitro* experiments) using a cyclone mill (Cyclotec, FOSS, Warrington, UK).

2.1.2. Ash

Samples were analysed for ash content by weighing 2 g of dried and ground feed into a pre-weighed porcelain crucible and ashed in a muffle furnace (Gallenkamp muffle furnace, Size 3, GAFSE 620, Gallenkamp, Loughborough, UK) to 550°C overnight. The ash remaining in the crucible was cooled in a desiccator to room temperature before being reweighed.

2.1.3. Crude protein

The nitrogen (N) content of feeds (concentrate, fresh and ensiled forages) was determined by the Dumas method (AOAC, 2012) using a Leco automatic analyser (FP-528 N; Leco Corp., St. Joseph, MI, USA) with EDTA as a standard (Sweeney, 1989). Approximately 0.15 g of oven dried (105 °C) and ground samples were weighed in an aluminium foil tray. Samples were heated to 1020 °C through a mixture of O_2 and CO_2 and the resulting N oxides reduced to N_2 by warmed copper fillings and the N measured with a thermal conductivity detector.

Samples of urea-treated whole crop wheat UWCW (Chapter 4) were analysed for N content by Kjeldhal digestion using a Tectator 1035 auto analyser (FOSS UK, Warrington, UK), due

to potentially excessive gaseous N losses during the drying procedure. Approximately 1.0 g of samples, after being milled in a grinder (Waring WSG30K Professional Spice Grinder, Leeds, UK), were weighed onto a Whatman No. 1 filter paper (Whatman plc, Maidstone, UK) and then placed in a 250 ml clean digestion tube. Following this, two tablets of kjeltab catalyst (3.5 g K_2SO_4 and 0.4 g $CuSO_4.5H_2O$; Thomson and Capper Limited, Runcorn, Cheshire, UK) were added. Exactly 16 ml of 98% (w/v) low nitrogen, sulphuric acid (Analar, VWR, Lutterworth, UK) was then added to each of the tubes. The samples were then digested on a heating block for 45 min. at 400 °C. Distilled water (75 ml) was added after allowing tubes to cool for 10 min. The estimation of nitrogen content was through back titration with 0.2 M hydrochloric acid.

2.1.4. Neutral detergent fibre

Neutral detergent fibre (NDF) content of the forages and concentrates was determined by a method adapted from Van Soest *et al*. (1991) using Fibertec apparatus (Tecator Fibertec 1020 Hot extractor, FOSS, UK Ltd, Warrington, UK). NDF determination was conducted with alpha-amylase and was corrected for ash.

2.1.5. Ether extract

Ether extract (EE) of dietary forages and concentrates from Chapters 3, 4, and 6 were determined in accordance with the solvent extraction method of MAFF (1986) using a Soxtec apparatus (FOSS, Warrington, UK).

2.1.6. pH determination

The pH of silage samples was determined in accordance with the method of MAFF (1986). Approximately 50 g of fresh silage was weighed into a 250 ml beaker and then 100 ml of distilled water added. The beaker was swirled for half a min every 15 min for 1 h. The resultant liquid was then filtered through a Whatman No. 1 filter paper (Whatman, Maidstone, UK) into another beaker and the pH was then determined using a pH probe (Jenway, Stone, Stafforshire). The probe was recalibrated daily using 2 pH solutions (colour key buffer solution yellow pH 7.0 and colour key buffer solution red pH 4.0) (VWR, International LTD, Poole, UK).

The pH of rumen fluid collected from animals slaughtered in a commercial abattoir in Chapters 3, 4, and 6, as well as vessels fluid in Chapters 5 and 7 were determined using the pH probe, as outlined above. The pH probe was recalibrated as described above. In Chapters 3, 4, and 6, on the morning of slaughter, the lambs were loaded for transport to the abattoir at about 0600 h. The journey to the abattoir was 40 minutes and the lambs were then slaughtered within 1.0 h. After slaughter, rumen fluid was directly collected in to 100 ml plastic pots and stored in a polystyrene box filled with ice. Rumen pH was determined (in the laboratory) within 1.0 h. after slaughter.

2.2. Blood sample collection

Blood samples were taken from lambs at 11:00 am via the jugular venepuncture using a 20 gauge 1.5" needle (Becton Dickinson Vacutainer Systems, Plymouth, UK) into 3 different plastic vacutainer tubes (Becton Dickinson Vacutainer Systems, Plymouth, UK).

Tubes containing K_2EDTA (10.8 mg/tube) were used for the determination of plasma trace element concentration. Tubes containing K_2EDTA (7.2 mg/tube) were used for the determination of whole blood haematology parameters and superoxide dismutase acidity (SOD). Tubes containing silica, which had been sprayed onto the inner walls of the tube to accelerate the clotting process, were used to collect blood serum for the determination of ceruloplasmin (Cp).

Blood samples for plasma were immediately centrifuged using a (Beckman Avanti 30) centrifuge at 1000 g for 15 min at a temperature of 4 ºC. The supernatant (plasma) was removed into 2 ml bijou tubes via disposable pipettes and stored at -20 ºC for subsequent trace element concentration analysis. Following the determination of haematology parameters in the second tubes, the remaining blood samples were pipetted with disposable pipettes into 2 ml bijou tubes (Sarstedt Ltd., Leicester, UK) and stored at -20 ºC for subsequent SOD analysis. Blood samples that were collected in the third set of tubes were left overnight in a refrigerator to coagulate and all tubes were then centrifuged at 1000 g for 15 min at a 4 ºC. The supernatant (serum) was then pipetted off with plastic pipettes into 2 ml bijou tubes and stored at – 20 ºC for subsequent Cp analysis. All stored whole blood, plasma, and serum samples before analyses were left at room temperature to defrost and then vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenstone, UK) to produce a uniform sample.

2.2.1. Haematology profile

The blood samples collected in the K_2EDTA (7.2 mg/tube) tubes were immediately analysed for haematology parameters, including white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), and haematocrit (Hct) using a Vet Animal Blood Counter (Woodly Equipment Company Ltd., Bolton, UK). Control blood (ABX Minotrol 16; Horiba ABX Diagnistic, Bedfordshire, UK) was used to calibrate the machine. The blood samples were mixed thoroughly for 15 min using a Spiramix 5 (Demley Insruments Ltd, West Sussex, UK) and then analysed for haematology parameters using the method described by Mackenzie *et al*. (1997) and Cope *et al*. (2009).

2.2.2. Plasma trace element determination

Prior to analysis, frozen plasma samples were defrosted to room temperature and vortexed thoroughly. Plasma samples were diluted in 1:20 in 0.5% of 70% HNO_3 (Fisher Scientific, Loughborough) with Ga (Qmx Laboratories, Thaxted, Essex, UK) added to each sample as an internal standard at 10 µg/L as described by Cope et al. (2009). Analysis was conducted using a calibration graph at concentration levels of 0, 50 µ/kg, 250 µ/kg, 500 µ/kg, 2500 µ/kg, and 5000 µ/kg in blank, standard 1, standard 2, standard 3, standard 4, standard 5 respectively. For Mo and Mn standard concentrations of 0, 5, 25, 50, 250, and 500 µ/kg respectively were used. All samples were analysed for Cu, Mo, Fe, Zn, and Mn using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Thermo Fisher Scientific Inc, Hemel Hempstead, UK).

2.2.3. Enzyme assays

2.2.3.1. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined using an adapted method of Misra and Fridovich (1977) for use on the Cobas Mira Plus (Ransod SD125, Randox Laboratories, County Antrim, UK). This method employed xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (I.N.T.) to form a red formazan dye. The SOD activity was then measured by the degree of inhibition of this reaction. Frozen heparinised blood samples were defrosted (to enhance lysing of cells) and vortexed using a MT-20 vortex-mixer (Philip Harris Ltd., Shenstone, UK). Into a 1 ml micro-centrifuge tube (Sarstedt Ltd., Leicester, UK), 250 µL of whole blood was pipetted and a further 750 µL of purite water was added. The sample was vortexed and 10 µL of this sample was then added to 490 µL of 0.01 mol/L phosphate buffer, pH 7.0 (Ransod, Ransod Laboratories, County Antrim, UK) into a micro-centrifuge tube and vortexed thoroughly. Samples were then transferred into Mira Cups (ABX diagnostics, Shefford, Bedfordshire, UK), and placed into reagent racks and analysed by an automated method on the Cobas Mira Plus (ABX diagnostics, Shefford, Bedfordshire, UK).

2.2.3.2. Cerulopasmin activity

Ceruloplasmin (Cp) activity was determined using an adapted method of Henry *et al*. (1974) for use on the Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). The ability of Cp to act as a general oxidase is utilised in this method, where Cp will oxidise p-phenylenediamine (PPD) to produce purple products that have an absorption peak between 530-550 nm. As PPD is also oxidised by any Cu or Fe present in the serum, a blank (CPB) must be run in which sodium azide inhibits the Cp activity, and the results subtracted from the test (CPT). Individual serum samples were pipetted into Mira cups (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the required reagent rack on the Cobs Mira Plus. A 0.1 M solution of PPD (BDH Laboratories Supplies, Poole, Dorset, UK) was prepared in 100 ml of 0.1 M acetate buffer and adjusted to pH 6.0. Sodium azide (BDH Laboratory Supplies, Pool, Dorset, UK) was prepared using 0.1% solution in pH 6.0 acetate buffer. A test (CPT) reagent was prepared by adding 20 ml acetate buffer pH 6.0 and 10 ml acetate buffer pH 6.0, 10 ml PPD solution pH 6.0 and 10 ml sodium azide solution. The activity of Cp (mg/dL) was calculated as:

Ceruloplasmin activity (mg/dL) = CPT- CPB

2.3. Live weight determination

Lambs were weighed once a week as specified in each experimental Chapter using a standard operating procedure with a weigh scale (IAE., Staffordshire, UK), and electronic display head by (Salter Bracknell LS300 electronic weigh scale, Staffordshire, UK). The scale was calibrated prior to use and between every 10 weighings using standard weights (F.J. Thornton and Co. Ltd., Wolverhampton, UK) for precision and accuracy.

2.4. Minerals determination of non-blood samples

2.4.1. Forage and concentrate minerals determination

Forages and concentrates were analysed for Cu, Mo, S, Fe, Zn, and Mn concentrations according to the method of Cope *et al*. (2009) and Sinclair *et al*. (2013:2017). Approximately 0.5 g of dried and ground sample was weighed into a 50 ml DigiTUBE (QMX Laboratories Ltd, Essex, UK), mixed with 6 ml of nitric acid (70%) (Fisher Scientific, Loughborough, UK) and 1 ml of 37% hydrochloric acid (HCl) (Fisher Scientific, Loughborough, UK), and placed into the DigiPREP (QMX Laboratories Ltd, Essex, UK) for digestion. Tubes (DigiTUBE) were heated to 45 °C and held for 1 minute before being increased to 65 °C and held for further 25 minutes. Then heat was increased to 100 °C and refluxed for 40 minutes using plastic watch glasses. Samples were cooled down to room temperature, and then made up to 50 ml with purite water and diluted in 1:20 in 2% HNO_3, 1% methanol, and 0.1 % Triton X-100 (Fisher Scientific, Loughborough, UK) prior to analysis by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS; Thermo Fisher Scientific Inc, Hemel Hempstead, UK).

2.4.2. Whole liver minerals determination

After slaughter, the entire liver was removed and placed into a zipped plastic bag, and weighed in order to determine whole liver weight. An approximately 0.250 g of fresh liver section (from the same place for all livers) were weighed into pre-weight 50 ml plastic tubes. Liver samples were oven-dried at 60 °C overnight. After this all tubes were removed from the oven into a desiccator and allowed to cool to room temperature in order to determine DM. Dried liver samples were digested with 6 ml of analytical reagent grade, 70% nitric acid (Fisher Scientific, Loughborough, UK) in an oven overnight at 60°C. Digested samples were removed from the oven and placed in a fume cupboard to reach room temperature. Samples were then made up to 50 ml with purite water and diluted (1:20) in 2% HNO3, 1% methanol, and 0.1 % Triton X-100 (Fisher Scientific, Loughborough, UK) prior to analyse by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) as described by Sinclair *et al*. (2013; 2017).

Chapter 3. The differences in copper metabolism of growing lambs fed dried grass pellets or maize silage supplemented without or with molybdenum and sulfur

3.1. Introduction

Copper-responsive disorders in animals can be caused by offering a diet deficient in Cu, or as a result of dietary interactions between Cu and antagonists, mainly Mo, S, and Fe that inhibit Cu absorption and metabolism (Phillippo *et al.*, 1987a, 1987b; Suttle, 1990; Suttle, 2010). The mechanism by which these minerals influence Cu haemostasis has been regarded as controversial and not fully understood over the years (Telfer *et al.*, 2004; Suttle, 2010). It has been shown that S present in the diet or from water reduces to sulfide, via rumen microorganisms within the anaerobic environment of the rumen, and then interacts with molybdate to produce a series of thiomolybdates (mono, di, tri, and tetra-thiomolybdates) that have a high affinity for Cu forming insoluble Cu-thiomolybdate complexes (Dick *et al.*, 1975; Suttle, 1991; Gould and Kendal, 2011). Thiomolybdates can be absorbed from the rumen, when insufficient dietary of Cu is available in the rumen or at high dietary Mo intake and very low Cu:Mo ratio (1:1) (Suttle, 2010), into the blood stream and binding with Cu-dependent enzymes such as ceruloplasmin (Cp), impairing their function (Price *et al.*, 1987; Gould and Kendal, 2011). A number of studies have confirmed that addition of Fe (250-1200 mg/kg DM) can also reduce Cu status in sheep (Prabowo *et al.*, 1988; Grace and Lee, 1990) and cattle (Bremner *et al.*, 1987; Phillippo *et al.*, 1987a). However, the antagonists' effect of Fe on Cu is not clear (Bremner *et al.*, 1987).

Dietary ingredient and forage type has been reported to have an effect on the degree of thiomolybdate formation although the mechanism for this effect is still not clear (Suttle, 1983a; Suttle, 2010). For instance, the coefficient of Cu absorption was found to be greater in grass hay and dried grass compared with grass silage or fresh grass (Suttle, 1980). Suttle (1983b) also demonstrated that the Cu absorption of conserved grass as hay was higher compared with fresh grass, and the antagonist effect of Mo on Cu availability was proportionately less when sheep were fed grass hay compared with fresh grass, with semi purified diets being intermediate (Suttle, 1983b). The reason for effects of forage type and preservation on Cu availability from forages are not clear, but may be related to the extent Cu releases from forages and subsequently to antagonists Suttle (1986), or difference in protein degradability between forages and the rate of sulfide production and CuxMoxS interactions, which in turn, affect Cu availability (Ivan, 1993).

Moreover, additional Mo and S has been found to have no effect on liver Cu status in dairy cows fed maize silage diet (Hart *et al*., 2011), whereas in dairy cows offered grass silage the addition of Mo and S markedly decreased liver Cu status (Sinclair *et al*., 2013). More recently, Sinclair *et al.* (2017) found that the effects of supplemental Mo and S were influenced by forage type. They showed that liver Cu status was higher in cows fed maize silage compared with those fed grass silage. In addition, the reduction in liver Cu concentration caused by the inclusion Mo and S in grass silage fed cows was greater compared with maize silage fed cows (Sinclair *et al.*, 2017). However, the reason for this effect was not clear. Previously, Suttle (1991) discussed that low rumen pH, caused by feeding diets containing highly fermentable carbohydrates, may improve Cu availability due to absorption of produced sulfide in the rumen, or break down of rumen thiomolybdates. There has been limited studies investigating the effects of forage type and antagonists level on the Cu metabolism in sheep. Therefore, the aim of this study was to investigate the effect of forage type (dried grass pellets or maize silage) either without or with added Mo and S on the performance and Cu metabolism in growing lambs.

3.2. Materials and methods

3.2.1. Animal procedures

All procedure involving animals were carried out according to the UK Animals (Scientific Procedures) Act 1986 and were approved by Harper Adams University Ethic Committee.

3.2.2. Animals and experimental design

A study was carried out at Harper Adams University (at 20th of June 2014) using 40 Texel-cross breed lambs with an initial mean body weight of 28.3 kg (s.e.d; 0.65) over a period of 8 weeks. Eight representative lambs were slaughtered immediately prior to the start of the study in a commercial abattoir, and liver samples were collected and stored at -20 °C prior to serve as a baseline for liver Cu levels. The remaining 32 lambs (male 12 and female 20) were blocked according to liveweight (LW) and sex, and then randomly allocated to one of four treatments, with eight lambs per treatment. The lambs were housed in a well-ventilated shed in individual pens and bedded on wood shavings. They had free accessed to water.

3.2.3. Diets

Lambs were fed a diet with a forage to concentrate ratio of 60:40 (DM basis) to meet their requirements to grow at 200 g/day (AFRC, 1993). The forages were either dried grass pellets (DGP) (Graze-on grass Pellets, Northern Crop Driers, Melrose Farm, Melbourne, York, UK) or maize silage (MS), which was made at Harper Adams University. Appropriate concentrates were formulated to produce isonitrogenous, isoenergetic diets (Table 3.1). The predicted metabolisable energy (ME) for experimental diets DGP and MS (60:40) was 11.60 and 11.70 MJ/kg DM respectively (AFRC, 1993).

Table 3.1. Raw material composition of the experimental concentrates (g/kg DM)

Ingredients, g/kg DM	Concentrate Diets[1]	
	DGP	MS
Barley	643	256
Sugar beet pulp (Shreds)	---	125
Soya bean meal	192	487
Molasses	73	74
Megalac	35	---
Mins/vits[1]	57	58
Total	1000	1000

[1] DGP= concentrate fed with dried grass pellets forage, MS= concentrate fed with maize silage.

[2] Mineral premix (25 kg/tonne) (Wynnstay Group P.L.C., Powys, Llansantffraid, UK). Major minerals (g/kg DM): Calcium, 236.2; Phosphorous, 20; Magnesium, 80; Sodium, 49.2. Trace elements (mg/kg DM); Iron, 3226; Iodine, 630; Cobalt, 120; Manganese, 8065; Zinc (chelates of amino acids), 2000; Zinc (oxide), 8057; Selenium (sodium selenite), 75.6; Selenium (Selenised yeast inactivated), 500. Vitamins; Vit A {E 672}, 400000 IU/kg; Vit D3 {E 671}, 80000 IU/kg. Vit E (all-rac-alpha-tocopheryl acetate) {3a700} 1500 mg/kg. Vit B12 cyanocobalamin 500 mcg/kg.

Chemical composition and mineral concentration of the forages and concentrates were chosen based on predicted values of MAFF (1992). Basal diets (DGP and MS; 60:40; forage: concentrate ratio at DM basis) were predicted to supply 2.86 g/kg DM of S and 2.85 mg/kg DM of Mo. To evaluate the effect of antagonists on Cu metabolism, lambs received diets that were either unsupplemented (-) or supplemented (+) with Mo at 3.15 mg/kg DM as ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Fisher Scientific, Leicester, UK), and S (1g/kg DM) as ammonium sulphate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA) to result in reducing Cu absorption by 50% (Suttle and MacLauchlan, 1976). The N content of the diets were balanced by the addition of 0.418 kg/tonne DM as feed grade urea (Trouw Nutrition, Cheshire, UK). Lambs were allocated by liveweight and sex to one of four dietary treatments (Table 3.2).

Table 3.2. Dietary treatments

Code	Treatments
DGP-	0.60 dried grass pellets:0.40 concentrate (DM basis), no addition antagonists
DGP+	0.60 dried grass pellets:0.40 concentrate (DM basis), with additional Mo and S
MS-	0.60 maize silage:0.40 concentrate (DM basis), no addition antagonists
MS+	0.60 maize silage:0.40 concentrate (DM basis), with additional Mo and S

Feed samples (forage and concentrates) were collected once weekly throughout the study. Then, at the end of the study, all feed samples were analysed for DM, Ash, CP, EE, NDF, and mineral contents as described in sections 2.1.1. to 2.1.5, and section 2.4.1 respectively. The chemical composition of the experimental diets are presented in Table 3.3.

Table 3.3. Analysed chemical and mineral composition of the experimental diets supplying 600 g/kg DM forage and 400 g/kg DM concentrates (60:40)[1].

Item	DGP-	DGP+	MS-	MS+
Chemical composition, g/kg DM				
DM, g/kg	899.1	899.5	559.2	559.6
CP,	166.7	166.3	146.4	142.1
EE,	22.1	23.0	20.1	20.9
NDF,	302.7	298.8	283.4	283.9
Ash,	85.2	87.9	68.0	66.2
Mineral composition, mg/kg DM				
Cu,	9.3	9.5	7.9	7.8
Mo,	1.9	4.6	2.7	4.8
S, g/kg DM	3.7	4.3	3.5	3.9
Fe,	458.5	462.1	263.3	256.5
Zn,	214.1	210.2	196.1	190.3
Mn,	198.9	193.7	155.7	158.2

[1] Diets consists of either dried grass pelleted (DGP) + concentrate or maize silage (MS) + concentrate at a ratio of 60:40 forage: concentrate. Diets DGP+ or MS+ received additional Mo and S, resulting in Mo ent 5 mg/kg DM and S content 4 g/kg DM.

3.2.4. Experimental routine

All lambs were offered fed twice a day at (08:30 and 16:30h). Forages (dried grass pellets and maize silage) were put into wooden troughs, and concentrates placed into plastic buckets. Feed refusals were collected twice a week (every Monday and Friday until the end of experiment) to estimate individual feed intake and feed conversion efficiency. The quantity of both diets offered was adjusted weekly according to the liveweight of the animal taken on the day of liveweight determination (section 3.2.4.2) to meet AFRC (1993) requirement. At the end of the study, lambs were sent to a commercial abattoir for slaughter. All lambs, including the eight representative lambs on day 0, were slaughtered following electrical stunning. Livers were collected immediately after slaughtering, weighed, and stored at -20°C for subsequent mineral content determination.

3.2.4.1. Blood sample collection

Blood samples were collected by jugular vein puncture (section 2.2.) once a week on a Tuesday at 11:00h for plasma and serum (sections 3.2.5). On weeks 0, 4, and 8 an additional EDTA tube was collected for haematology analysis and an aliquot stored at -20°C for SOD analysis (section 3.2.5). Blood samples (week 0) were collected at 20th of June 2014.

3.2.4.2. Liveweight determination

Lambs were weighed once a week on Friday at 11:00 using Standard Operating Procedure as described in section 2.3. Daily liveweight gain (DLWG) was calculated using regression analysis.

3.2.5. Blood analysis

Fresh blood samples after being collected were analysed immediately for haematocrit (Hct), haemoglobin concentration (Hb), red blood cell counts (RBC), and while blood cell counts (WBC) using using a Vet Animal Blood Counter (section 2.2.1). Frozen samples of whole blood, plasma, and serum were defrosted thoroughly at room temperature. Whole blood samples were analysed for SOD activity using a Cobas mira plus as described in section 2.2.3.1. Plasma samples were used to determine mineral concentrations (section 2.2.2), and trichloroacetic acid soluble concentration (section 2.2.2.1). Blood serum samples were also analysed for ceruloplasmin activity (Cp) using a Cobas mira plus (section 2.2.3.2).

3.2.6. Liver mineral analysis

Liver samples were analysed for mineral concentrations using an ICP-MS as described in section 2.4.2. Whole liver minerals content was determined by multiplying liver mineral concentrations by liver weight and liver DM. Liver minerals retention was determined by substracting whole liver minerals content of the initial slaughter group from final whole liver mineral content and divided by days of the whole study period, which was 8 weeks.

3.2.7. Rumen pH determination

Rumen fluid samples were collected immediately after slaughter of the lambs, put into 100 ml plastic pots and stored on ice prior to measuring pH within an hour after slaughtering (section 2.1.6).

3.2.8. Statistical analysis

Performance, plasma minerals, haematology, and enzyme activities were analysed by repeated-measures ANOVA as a 2x2 factorial randomise block design with the main effects of forage type (F), antagonists (Ant.), and interaction between forage type and antagonists (Int.). Daily Liveweight gain (DLWG) was calculated by regression analysis and analysed by ANOVA. For plasma Cu concentration, Cp activity, Cp:Pl-Cu ratio, SOD activity, haemoglobin concentration, WBC counts, week zero was used as a covariate. All statistical analysis were conducted using Genstat version 17.1 (Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Significance was set at $P < 0.05$ and trends at $P < 0.10$. Significant differences between means were tested using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

3.3. Results

3.3.1. Health observation

Additional Mo and S in the current study did not produce clinical symptoms, and all lambs were healthy and none were removed from the study.

3.3.2. Animal performance and intake

There was no effect ($P>0.05$) of forage x antagonist on DLWG, DM intake, FCE (Table 3.4), and weekly liveweight (Table 3.5) of the lambs throughout the study. There was also no effect ($P>0.05$) of the antagonists on these parameters. However, lambs fed the maize silage were heavier ($P<0.05$) from week 3 until the end of the study compared with lambs fed the dried grass pellets. The DMI was not different ($P>0.05$) between lambs fed maize silage or dried grass pellets (Table 3.4). Whereas, the maize silage fed lambs had a higher ($P<.001$) DLWG compared with the dried grass pellets fed lambs. The DLWG for maize silage treatment groups was almost the same as predicted at 200 g/d as calculated by AFRC (1993), while in the dried grass pellet treatment lambs was 50 g/d lower than the predicted value. The FCE was also higher ($P<0.001$) in lambs on maize silage diet compared with those on dried grass pellets.

Table 3.4. Intake and performance of growing labs fed diets containing dried grass pellets (DGP) or maize silage (MS) supplemented either without (-) or with (+) Mo and S[1].

Items	Treatment				Significance			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Intake, kg/d								
Forage DMI,	0.56	0.55	0.55	0.57	0.016	0.530	0.888	0.145
Concentrate DMI,	0.39	0.39	0.39	0.39	0.007	0.510	0.984	0.653
Total DMI,	0.95	0.94	0.95	0.97	0.022	0.496	0.924	0.223
DLWG, kg/d	0.15	0.15	0.20	0.19	0.016	<.001	0.794	0.484
FCE[2]	0.15	0.16	0.22	0.20	0.015	<.001	0.711	0.293

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists.

[2] FCE calculated as DLWG (kg/d) divided by DMI (kg/d)

Table 3.5. Liveweight in growing lambs fed diets dried grass pellets (DGP) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Week	Treatment				Significance[2]			
	DGP-	DGP+	MS -	MS +	s.e.d	F	A	Int.
0	28.2	28.2	28.4	28.4	0.48	0.584	1.000	1.000
1	28.2	27.2	28.6	28.4	0.77	0.166	0.288	0.464
2	30.1	29.3	29.1	29.4	1.08	0.571	0.746	0.468
3	31.2	30.3	32.1	32.3	0.88	0.034	0.585	0.401
4	32.3	31.6	33.4	33.5	0.93	0.030	0.674	0.607
5	33.3	32.8	34.7	34.6	0.98	0.031	0.688	0.823
6	34.0	33.8	35.9	36.1	1.03	0.009	0.966	0.767
7	34.9	34.1	37.5	37.0	0.99	<.001	0.380	0.859
8	35.9	35.9	39.0	38.3	1.08	0.002	0.600	0.657

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of differences.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

3.3.3. Mineral intake

There was no forage x antagonist interaction (P>0.05) on Cu, S, Fe, Zn, and Mn intake (Table 3.6). However, there was a forage x antagonist interaction (P<0.05) on Mo intake, which was higher in the lambs fed maize silage diet supplemented with Mo and S, followed by those fed dried grass pellet diet supplemented with antagonists compared with those fed maize silage unsupplemented with Mo and S, with the lowest Mo intake in the lambs fed dried grass pellets unsupplemented with Mo and S. The Mo and S supplemented lambs had a higher (P<0.001) S intake compared with the lambs unsupplemented with Mo and S, with mean values of 3.88 and 3.46 mg/d respectively. There was no effect (P>0.05) of additional Mo and S on Cu, Fe, Zn, and Mn intake. The dried grass fed lambs had a higher (P<0.001) Cu, S, Fe, Zn, and Mn intake compared with the maize silage fed lambs, but Mo intake was higher (P<0.001) in maize silage fed lambs than the dried grass pellets fed lambs.

Table 3.6. Mineral intake in growing lambs fed diets containing dried grass pellets (DGP) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Mineral, mg/d	Treatment				Significance[2]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
Cu	8.85	8.88	7.46	7.59	0.180	<.001	0.55	0.702
Mo	1.85[a]	4.42[c]	2.63[b]	4.74[d]	0.077	<.001	<.001	<.001
S	3.57	4.01	3.35	3.76	0.082	<.001	<.001	0.781
Fe	437.3	432.6	248.9	247.8	7.32	<.001	0.575	0.733
Zn	201.4	196.8	179.7	183.8	2.99	<.001	0.939	0.160
Mn	189.7	187.0	148.1	152.8	2.60	<.001	0.716	0.166

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of differences.

[2] [a,b,c,d] Means within a row with different superscripts are significantly different (P<0.05).

3.3.4. Rumen pH

There was no forage x antagonist interaction (*P*>0.05) on rumen pH. There was also no effect (*P*>0.05) of the antagonists on rumen pH (Table 3.7). However, lambs offered dried grass pellets had a lower (*P*<0.05) rumen pH compared with those offered maize silage, with the mean values of 6.15 and 6.47 respectively.

Table 3.7. Rumen pH of growing labs fed diets containing dried grass pellets (DGP) or maize silage (MS) supplemented either without (-) or with (+) Mo and S.

	Treatment				Significance[1]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Rumen pH	6.19	6.12	6.47	6.47	0.156	0.010	0.728	0.762

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of differences.

3.3.5. Liver mineral status

3.3.5.1. Liver mineral concentrations

The mean liver concentration of Cu, Mo, Fe, Zn and Mn of the representative lambs at the beginning of the experiment is presented in Table 3.8. There was no forage x antagonist interaction (P>0.05) on liver mineral concentration (Table 3.9). There was also no effect (P>0.05) of Cu antagonists on liver Mo, Fe, Zn, and Mn concentration. Lambs fed diets supplemented with antagonists had a lower (P<0.05) liver Cu concentration compared with those not receiving antagonists, with mean values of 190 and 292 mg/kg DM (s.e.d, 40.1) respectively.

The dried grass pellets fed lamb had a higher (P<0.05) liver Cu and Fe concentration compared with the maize silage fed lambs. However, forage type had no effect on liver Mo, Zn, and Mn concentrations (P>0.05).

Table 3.8. Liver mineral concentration of (8) representative lambs slaughtering at the starting of the study.

Minerals, mg/kg DM	Liver mineral concentrations	Standard Deviation
Cu,	173.6	± 31.4
Mo,	3.8	± 0.8
Fe,	351	± 67.3
Zn,	103.6	± 15.1
Mn,	20.7	± 6.1

Table 3.9. Liver mineral concentration in growing lambs fed diets containing dried grass pellets (DGP) or maize silage (MS) fed either without (-) or with (+) Mo and S.

Minerals, mg/kg DM	Treatment				Significance[1]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Cu,	358	237	224	142	56.7	0.010	0.019	0.636
Mo,	4.46	4.26	4.32	4.23	0.240	0.635	0.400	0.738
Fe,	371.8	350.5	304.4	317	27.62	0.017	0.827	0.394
Zn,	127.2	123.3	107.6	128	15.22	0.495	0.450	0.270
Mn,	24.30	19.00	24.90	24.80	4.470	0.327	0.403	0.411

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of differences.

3.3.5.2. Whole liver mineral content

There was no forage x antagonist interaction (*P*>0.05) on whole liver Cu content (Table 3.10). However, lambs offered dried grass pellets had a higher (*P*<0.05) whole liver Cu content compared with those offered maize silage, with mean values of 48.7 and 32.6 mg/liver (s.e.d, 6.6) respectively. Similarly, lambs unsupplemented with antagonists had a higher (*P*<0.05) liver Cu contentment compared with those supplemented with Mo and S, with mean values of 48.6 and 32.7 mg/liver (s.e.d, 6.6) respectively. Dietary treatment had no (*P*>0.05) effect on whole liver content of Mo, Fe, Zn, and Mn.

Table 3.10. Whole liver mineral contents[1] in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) Mo and S.

Minerals, mg/liver	Treatment					Significance[2]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Cu,	57.2	40.2	40.0	25.2	9.33	0.024	0.025	0.867
Mo,	0.72	0.71	0.77	0.75	0.051	0.194	0.719	0.914
Fe,	59.8	58.2	54.5	55.7	5.00	0.287	0.964	0.692
Zn,	20.4	20.5	19.2	22.4	2.49	0.840	0.361	0.379
Mn,	3.87	3.20	4.38	4.31	0.682	0.107	0.450	0.543

[1] whole liver minerals content = whole liver weight x liver DM x final liver Cu concentration (mg/kg DM).

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

3.3.5.3. Liver mineral retention

There was no forage x antagonist interaction (*P*>0.05) on whole liver retention (Table 3.11) for all minerals. Compared to the total liver Cu content in the initial slaughter group, lambs fed the dried grass pellets and the maize silage without antagonists, or dried grass pellets with antagonists all had a net positive retention, except lambs fed the maize silage diet supplemented with antagonists which had a negative retention and lost 0.07 mg Cu/kg DM per day. Lambs fed dried grass pellets had a lower liver Cu retention compared with the lambs fed maize silage, when Mo and S were included (*P*<0.05). There was no effect (*P*>0.05) of dietary treatment on liver retention of Mo, Fe, Zn, and Mn.

Table 3.11. Liver mineral retention in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) Mo and S[1].

Minerals, µg/d	Treatment				Significance[2]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Cu, mg/d	0.50	0.20	0.19	-0.07	0.167	0.024	0.025	0.867
Mo,	2.05	1.89	2.99	2.68	0.911	0.194	0.719	0.914
Fe,	73	45	-21	1.0	89.4	0.287	0.964	0.692
Zn,	68	69	46	103	44.5	0.840	0.361	0.379
Mn,	11.00	-0.90	20.20	18.90	12.180	0.107	0.450	0.543

[1] Liver minerals retention were calculation by substrate whole liver minerals content at day zero from final whole liver Cu content divided by whole study period (days).

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

3.3.6. The mean of plasma mineral profile, Cu-mediated enzymes, and haematology profile

Repeated measures analysis indicates that there was no effect (P>0.05) of forage type, the addition of Mo and S, or forage type x antagonist interaction on the mean plasma Cu, Mo, Fe, and Zn concentrations (Table 3.12). There was also no effect (P>0.05) of dietary treatment on the mean Cp activity, Cp:Pl-Cu ratio, or SOD activity. Similarly, no effect (P>0.05) was observed of dietary treatment on the mean Hct (%), Hb concentration, RBC counts, or WBC counts.

Table 3.12. Effect of forage type dried grass pellets (DGP) and maize silage (MS) fed without (-) or with (+) added Mo and S on mean indicators of blood Cu status over the study period of lambs[1].

Items	Treatments				Significance			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
Cu, µmol/L	16.5	16.5	16.5	16.6	1.20	0.933	0.953	0.938
Mo, µmol/L	0.38	0.39	0.41	0.54	0.184	0.115	0.223	0.279
Fe, µmol/L	45.2	45.4	51.0	49.3	7.80	0.029	0.730	0.647
Zn, µmol/L	12.9	13.0	11.9	11.5	1.02	0.037	0.763	0.645
Cp, mg/dL	12.0	11.7	12.4	11.9	1.54	0.711	0.524	0.850
Cp:Pl-Cu	0.74	0.74	0.75	0.73	0.091	0.924	0.650	0.758
SOD, U/g of Hb	2384	2463	2529	2428	184.5	0.702	0.895	0.444
Hct, %	31.7	30.5	31.3	29.7	1.52	0.470	0.078	0.749
Hb, g/dL	11.9	11.6	11.7	11.2	0.55	0.398	0.158	0.820
RBC, 10^6/mm^3	11.7	11.1	11.2	11.0	0.56	0.342	0.195	0.542
WBC, 10^3/mm^3	11.1	11.9	10.5	9.6	1.50	0.111	0.895	0.396

[1] week 0 values were used as a covariate where appropriate.

[2] SOD= superoxide dismutase activity; Hct- haematocrit; Hb= haemoglobin; RBC= red blood cells; WBC= white blood cells.

[3] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

3.3.7. Plasma mineral concentrations

3.3.7.1. Plasma copper concentration

There was an effect (*P*<0.001) of time, with plasma concentrations declining over the period of the study. There was also a time x forage x antagonist interaction (*P*<0.05) on Pl-Cu concentration. However, there was no time x forage interaction, or time x antagonist interaction (*P*>0.05) on Pl-Cu concentration.

There was no effect (*P*>0.05) of dietary treatment on Pl-Cu concentration from week 1 until week 7 (Table 3.13). However, at week 8, there was a forage x antagonist interaction (*P*=0.015) on Pl-Cu concentration. At week 8, the highest Pl-Cu concentration was in the lambs fed dried grass pellets and maize silage supplemented without and with antagonists respectively, and the lowest Pl-Cu concentration was in lambs fed dies grass pellets or maize silage supplemented with or without antagonists respectively. There was no effect (*P*>0.05) of forage type or antagonists on Pl-Cu concentration at any weekly time points.

Table 3.13. Plasma copper concentration in growing lambs fed diets dried containing grass pelleted (DGP) or maize silage (MS) supplemented without (-) or with (+) Mo and S (µmol/L)[1].

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
0	21.2	19.5	23.8	19.5	2.17	--	--	--
1	18.8	20.9	20.5	19.3	1.21	0.972	0.495	0.066
2	15.9	17.4	16.9	17.7	0.95	0.271	0.098	0.598
3	12.9	13.8	13.7	13.3	0.83	0.758	0.591	0.258
4	15.1	14.3	15.7	15.0	0.82	0.247	0.217	0.924
5	17.7	17.5	16.4	17.0	1.97	0.512	0.881	0.792
6	16.1	14.0	15.6	15.1	0.91	0.715	0.054	0.249
7	15.9	15.8	14.8	15.3	1.06	0.318	0.848	0.672
8	15.7^{b}	14.4^{a}	14.3^{a}	15.7^{b}	0.73	0.946	0.963	0.015

[1] week 0 used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.534	<.001
Time x Forage effect	0.464	0.504
Time x Antagonist effect	0.496	0.261
Time x Forage x Antagonist effect	0.846	0.045

3.3.7.2. Plasma molybdenum concentration

There was an effect (*P*<.001) of time on Pl-Mo concentration. However, there were no time x treatment interaction (*P*>0.05) on Pl-Mo concentration.

There was no forage x antagonist interaction (*P*>0.05) on Pl-Mo concentration at any weekly time point. At week 1, and from week 4 onwards, lambs fed MS had a higher (*P*<0.05) Pl-Mo concentration compared with those fed dried grass pellets (Table 3.14). From week 1 until week 8, lambs offered diets supplemeted with Mo and S had a higher (*P*<0.05) Pl-Mo concentration compared with those offered diets no added Mo and S.

Table 3.14. Plasma molybdenum concentration of growing lambs fed diets containing forages dried grass pelleted (DGP) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

Week	Treatment				Significance			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
0	1.63	0.28	1.00	1.08	0.494	0.854	0.193	0.142
1	0.30	0.44	0.45	0.59	0.087	0.021	0.034	0.995
2	0.24	0.36	0.29	0.39	0.045	0.230	0.003	0.696
3	0.21	0.28	0.25	0.33	0.033	0.055	0.003	0.958
4	0.23	0.29	0.34	0.48	0.055	<.001	0.015	0.342
5	0.28	0.37	0.40	0.62	0.066	<.001	0.004	0.204
6	0.21	0.28	0.31	0.45	0.046	<.001	0.003	0.238
7	0.19	0.31	0.33	0.47	0.056	0.001	0.003	0.762
8	0.17	0.25	0.28	0.40	0.040	<.001	0.002	0.570

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.088	<.001
Time x Forage effect	0.056	0.414
Time x Antagonist effect	0.056	0.291
Time x Forage x Antagonist effect	0.130	0.306

3.3.7.3. Plasma iron concentration

There was an effect (*P*<0.001) of time on Pl-Fe concentration, with concentrations decreasing over a period of the study. However, there was no time x treatment interaction (*P*>0.05) on Pl-Fe concentration.

There was no forage x antagonist interaction (*P*>0.05) on Pl-Fe concentration throughout the study (Table 3.15). There was also no effect (*P*>0.05) of antagonists on Pl-Fe concentration. However, during week 7 and 8 lambs fed maize silage had a higher (*P*<0.05) Pl-Fe concentration compared with those fed dried grass pellet.

Table 3.15. Plasma iron concentration of growing lambs fed diets containing forages dried grass pelleted (DGP) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S[1] (µmol/L).

Week	Treatment				Significance[2]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	69.3	65.3	59.9	71.4	8.71	0.789	0.544	0.223
1	58.4	61.8	77.5	63.7	7.99	0.077	0.365	0.144
2	45.7	44.6	70.9	60.9	14.17	0.051	0.584	0.659
3	40.4	40.4	44.0	48.0	6.60	0.248	0.674	0.673
4	38.7	38.4	39.1	39.3	3.75	0.814	0.978	0.920
5	45.7	47.5	45.2	39.1	9.11	0.499	0.740	0.546
6	37.8	37.2	36.3	41.4	5.37	0.733	0.565	0.465
7	38.8	39.0	47.1	44.1	2.03	<.001	0.326	0.286
8	31.8	32.8	37.5	36.0	2.74	0.032	0.901	0.519

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d.	P-value
Time effect	3.83	<.001
Time x Forage effect	2.08	0.097
Time x Ant. Effect	2.08	0.814
Time x Forage x Ant. Effect	5.51	0.608

3.3.7.4. Plasma zinc concentration

There was an effect (*P*<0.001) of time on Pl-Zn concentration. However, there was no time x treatment interaction (*P*>0.05) on Pl-Fe concentration.

There was no effect (*P*>0.05) of dietary treatment on Pl-Zn concentration at any weekly time points (Fig. 3.1).

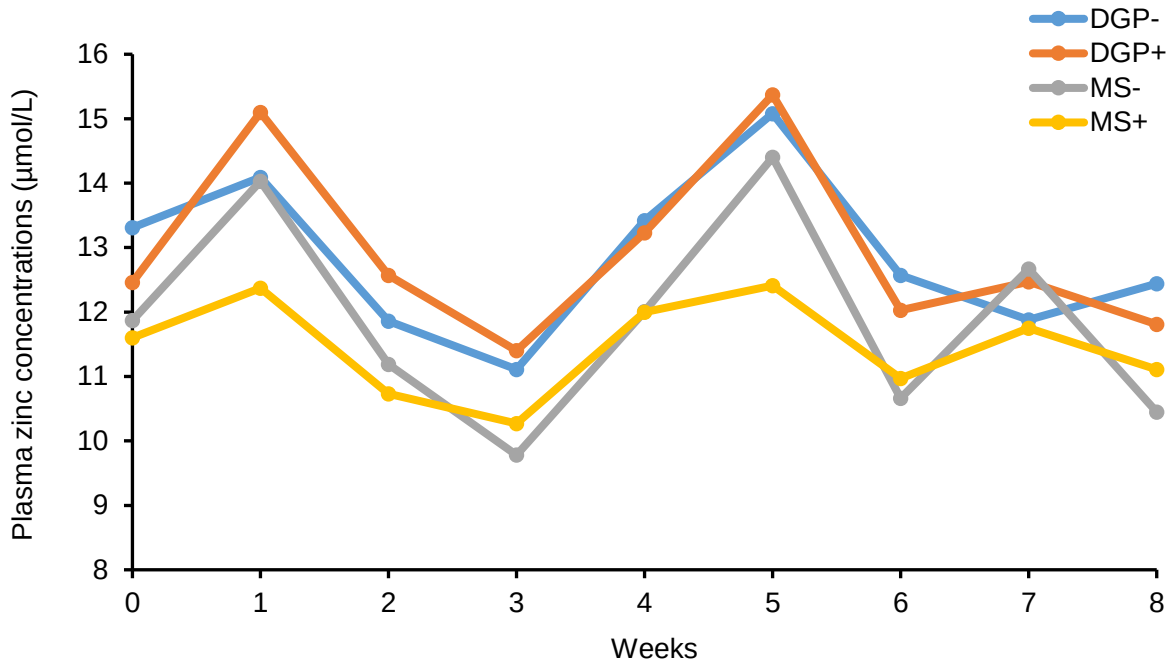


Figure 3.1. Plasma Zinc concentration in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) supplemented without (-) or with (+) Mo and S. Error bars indicate s.e.d. Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.3517	<.001
Time x Forage effect	0.5519	0.398
Time x Antagonist effect	0.5519	0.731
Time x Forage x Antagonist effect	0.7242	0.090

3.3.8. Ceruloplasmin activity

There was an effect (*P*<0.001) of time on Cp activity, with activity decreasing over a period of the study. However, there was no time x treatment interaction (*P*>0.05) on Cp activity.

There was no effect (*P*>0.05) of dietary treatment on Cp activity at any weekly time points (Fig. 3.2).

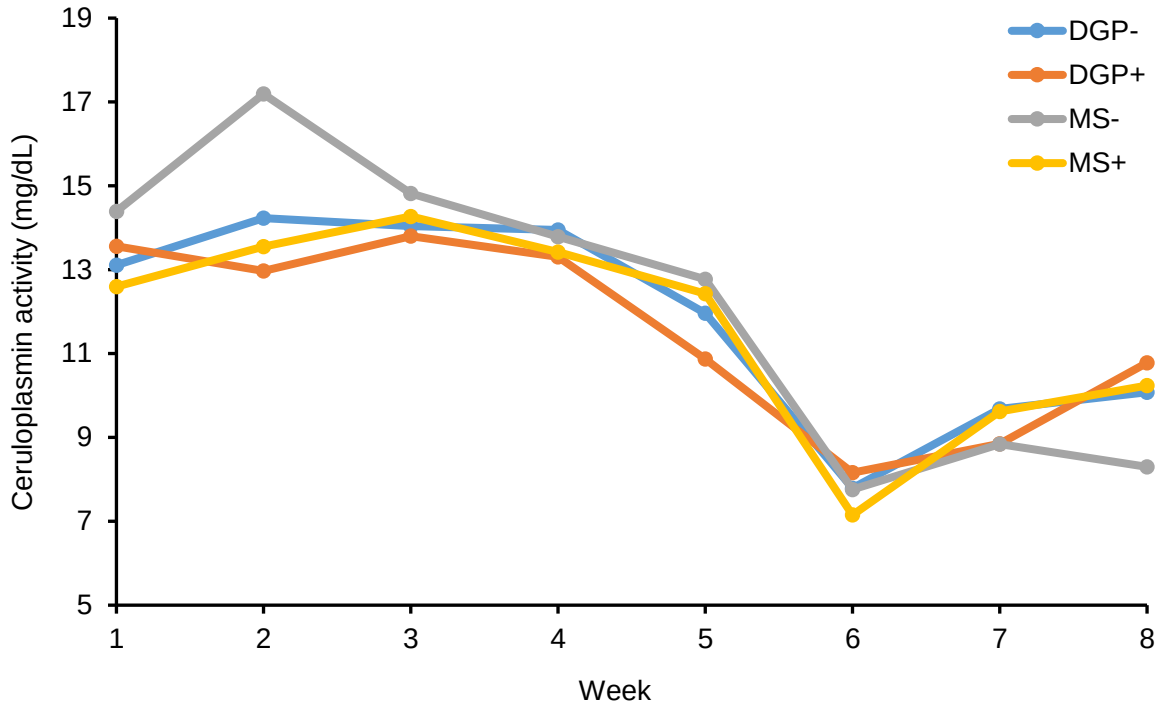


Figure 3.2. Ceruloplasmin activity of growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) supplemented without (-) or with (+) Mo and S. Week 0 values were used as a covariate. Error bars indicate s.e.d. Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.689	<.001
Time x Forage effect	0.595	0.439
Time x Antagonist effect	0.615	0.315
Time x Forage x Antagonist effect	1.086	0.782

3.3.9. Ceruloplasmin to plasma copper ratio

There was an effect (*P*<0.001) of time on Cp:Pl-Cu ratio. However, there was no time x treatment interaction (*P*>0.05) on Cp:Pl-Cu ratio.

There was no effect (*P*>0.05) of dietary treatment, from week 0 until week 7, on Cp:Pl-Cu ratio (Table 3.16). However, at week 8, lambs fed diets supplemented with Mo and S had a higher (*P*<0.05) Cp:Pl-Cu ratio compared with those not receiving Mo and S, with the mean values of 0.73 and 0.62 (s.e.d, 0.050) respectively.

Table 3.16. Ceruloplasmin to plasma copper ratio in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) Mo and S[1].

Week	Treatment[2]				Significance[3]			
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int.
0	0.65	0.53	0.69	0.66	0.102	--	--	--
1	0.69	0.70	0.66	0.69	0.061	0.705	0.749	0.867
2	0.91	0.77	0.97	0.81	0.101	0.594	0.054	0.896
3	1.07	1.05	1.08	1.07	0.132	0.910	0.886	0.910
4	0.94	0.89	0.90	0.89	0.137	0.856	0.784	0.835
5	0.69	0.68	0.83	0.74	0.102	0.180	0.472	0.605
6	0.48	0.57	0.51	0.47	0.064	0.421	0.599	0.173
7	0.62	0.58	0.61	0.63	0.061	0.633	0.813	0.508
8	0.64	0.81	0.60	0.64	0.070	0.081	0.049	0.196

[1]Week 0 values were used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.043	<.001
Time x Forage effect	0.032	0.346
Time x Antagonist effect	0.032	0.329
Time x Forage x Antagonist effect	0.065	0.838

3.3.10. Superoxide dismutase activity

There was no effect (*P*>0.05) of time on superoxide dismutase activity. However, there was time x treatment interaction (*P*<0.05) on SOD activity (*P*>0.05).

There was no effect (*P*>0.05) of dietary treatment on blood SOD activity throughout the study (Table 3.17), except at week 8. At week 8, there was forage x antagonist interaction (*P*<0.05) on on SOD activity, with the highest value in the lambs fed dried grass pellet supplemented with antagonists compared with those fed other diets.

Table 3.17. Superoxide dismutase activity (SOD) in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) added Mo and S (U/ g Hb)[1].

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	2229	1974	1824	1843	190.0	--	--	--
4	2521	2371	2536	2530	217.6	0.541	0.622	0.640
8	2248[a]	2555[b]	2523[ab]	2326[ab]	147.5	0.966	0.667	0.023

[1] week 0 values used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists. s.e.d= standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	61.0	0.254
Time x Forage effect	123.0	0.453
Time x Antagonist effect	114.5	0.360
Time x Forage x Antagonist effect	132.0	0.020

3.3.3.11. Haematology parameters

3.3.11.1. Haematocrit

There was no effect (P>0.05) of time or time x treatment interaction on blood haematocrit (%).

At week 4, lambs fed diet supplemented with Mo and S had a lower Hct (%) compared with those fed diet no added Mo and S (P<0.05), with mean values of 29.9 and 31.9 (s.e.d, 1.197) respectively (Table 3.18). However, at week 8, dietary treatment had no effect on blood Hct (%) (P>0.05).

Table 3.18. Haematocrit (%) in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) added Mo and S[1].

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	28.83	32.74	33.95	31.05	2.818	--	--	--
4	31.79	31.42	31.93	28.38	1.268	0.100	0.034	0.103
8	31.51	29.56	30.76	30.92	1.740	0.760	0.473	0.418

[1] week 0 values used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists. s.e.d=standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.734	0.800
Time x Forage effect	0.783	0.135
Time x Antagonist effect	0.770	0.416
Time x Forage x Antagonist effect	1.070	0.278

3.3.11.2. Haemoglobin concentration

There was no time or time x treatment interaction on haemoglobin concentration (*P*>0.05).

At week 4, lambs offered dried grass pellets had a higher Hb (*P*<0.05) concentration compared with those offered maize silage, with the mean value of 11.94 and 11.29 g/dL (s.e.d, 0.223) respectively (Table 3.19). At week 8, there was no effect of dietary treatment on blood Hb concentration (*P*>0.05).

Table 3.19. Haemoglobin concentration (g/dL) in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) added Mo and S[1].

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	11.56	13.38	13.99	12.29	1.206	--	--	--
4	11.90	11.98	11.70	10.87	0.323	0.006	0.103	0.072
8	11.93	11.14	11.77	11.60	0.688	0.718	0.322	0.553

[1] week 0 values used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists. s.e.d=standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.244	0.990
Time x Forage effect	0.295	0.063
Time x Antagonist effect	0.291	0.849
Time x Forage x Antagonist effect	0.381	0.412

3.3.11.3. Red blood cell counts

There was no time or time x treatment interaction on RBC counts (*P*>0.05).

There was no forage x antagonist interaction (*P*>0.05) on RBC counts throughout the study (*P*>0.05). At week 4, lambs offered dried grass pellets tended (*P*<0.1) to have higher RBC counts compared with the lambs offered maize silage, with mean values of 11.58 and 11.09 $10^6/mm^3$ (s.e.d, 0.248) respectively (Table 3.20). During week 4 lambs offered diets supplemented with Mo and S also had a lower RBC counts (11.04 $10^6/mm^3$) compared to those offered diets unsupplements with Mo and S (11.63 $10^6/mm^3$) (s.e.d, 0.246) (*P*<0.05).

Table 3.20. Red blood cell counts ($10^6/mm^3$) in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) added Mo and S.

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	10.96	12.27	12.49	11.79	0.996	--	--	--
4	11.76	11.40	11.50	10.68	0.354	0.061	0.024	0.378
8	11.72	10.86	10.96	11.35	0.708	0.836	0.667	0.237

[1] week 0 values used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.248	0.656
Time x Forage effect	0.307	0.325
Time x Antagonist effect	0.304	0.375
Time x Forage x Antagonist effect	0.393	0.250

3.3.11.4. White blood cell counts

There was no time or time x treatment interaction on white blood cell counts (*P*>0.05).

At week 4 and 8, there was also no effect (*P*>0.05) of dietary treatment on WBC count (Table 3.21).

Table 3.21. White blood cell counts (10^3/mm^3) in growing lambs fed diets containing dried grass pelleted (DGP) or maize silage (MS) fed either without (-) or with (+) added Mo and S[1].

Week	Treatment[2]					Significance[3]		
	DGP-	DGP+	MS-	MS+	s.e.d	F	A	Int
0	9.83	13.58	13.74	11.20	2.023	--	--	--
4	11.34	11.69	11.42	8.78	1.501	0.167	0.254	0.200
8	10.88	12.03	9.63	10.44	1.571	0.196	0.376	0.886

[1] week 0 values used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forage type and antagonists.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonists, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.580	0.920
Time x Forage effect	0.872	0.937
Time x Antagonist effect	0.870	0.088
Time x Forage x Antagonist effect	1.045	0.156

3.4. Discussion

In the current study, lambs on MS were heavier than those on DGP from week 3 onwards. Ware and Zinn (2005) and Salinas-Chavira *et al.* (2013) observed a depression in weight gain of steers fed pelleted diets compared with straw. The basis of this effect was attributed to reduce diet acceptability when offered as a pelleted diet (Salinas-Chavira *et al.*, 2013). However, the feeding system in this study was restrictive, and there was no refusal in DGP throughout the study. The lower FCE in dried grass pellets fed lambs compared with maize silage reflects the poorer energy utilisation with dried grass pellets than maize silage. Boucque *et al.* (1973) found that the digestibility (determined with wethers) and the net energy content of whole plant maize-pellets were generally lower than those of whole plant maize silages, resulting in a more efficient utilisation of the DM of whole plant maize silages as compared to the pelleted form. Thomson and Beever (1980) showed that less organic matter and energy are digested in the rumen, and that total apparent digestibility of pelleted forage decreases as a result of an increased rate of fermentation and fractional outflow of particulate matter from the rumen. Thomson and Beever (1980) also reported that the depression in overall apparent digestibility of organic matter in the case of grasses is generally greater (up to 15 %) compared with legumes (3-6 %), which was attributed to the potentially higher structural carbohydrate content of grasses. Moreover, Knaus *et al.* (1999) showed low efficiency of utilisation of energy consumed in pellet-fed Simmental dairy cows compared with maize silage-fed cows was associated with the reduction in digestibility of forages (grass clover and whole plant maize) as a consequence of the alteration of particle size distribution by pelleting. It has been reported that increasing NDF from 40 to 80 g/kg DM resulted in diluting dietary energy and reduced gain efficiency and dietary net energy (Salinas-Chavira *et al.*, 2013). This response is consistent with the higher NDF content in DGP compared with the MS.

Replacing grass silage with maize silage has been reported to result in increased performance of dairy cattle and sheep (Keady *et al.*, 2013). Keady *et al.* (2007) reported that replacing grass silage by maize silage significantly increased (20%) DLWG in beef cattle, and this improvement in performance was attributed to the improvement in utilisation of metabolisable energy. Likewise, Keady and Hanrahan (2009) demonstrated that ewes on MS had a higher liveweight compared with grass silage (63 and 61.2 kg) respectively. Therefore, other factors such as the lower efficiency of energy utilisation, and lower digestibility in DGP compared with MS may have contributed to the lower liveweight in the DGP fed lambs compared with the MS fed lambs.

In the present study, lambs fed DGP had a lower rumen pH compared to those on MS. Low rumen pH in lambs fed DGP compared to those fed MS diet is probably associated with smaller feed particle size of the DGP which influences the buffering capacity of the rumen. Pelleting forage has been found to depress rumen pH (Dafaalla and Kay, 1980) potentially caused by the shorter time spent eating and ruminating pelleted forage (Ørskov, 1987; Minson, 1990). Recently, Bofonate *et al.* (2016) demonstrated the shorter particles in a pelleted diet compared to a TMR offered to cows resulted in decreased rumination time and total potential digestibility of NDF, possibly as a consequence of an increased the rate of passage from the rumen by pelleting diet, limiting potential degradation.

The addition of dietary Mo and S had no significant effect on the lamb's performance characteristics. These results are in agreement with the results of Williams (2004) and Alimon *et al.* (2011). Williams (2004) reported that lambs receiving diets containing Mo at 5 or 10 mg/kg DM (for 70 days) had no significant effect on lamb's performance. Similarly, Suttle (2012) also did not find any significant effect of Mo and S on liveweight when 2 mg/kg DM of Mo and 3 g/kg DM of S were added to Texel lambs diet for 96 days. However, Humphries (1983) and Phillippo *et al*. (1987a;1987b) reported that additional Mo at 5 mg/kg DM reduced growth rate in heifers after 16 weeks of feeding. However, the reason for the antagonist impacting on growth rate was not clear, but in both Humphries *et al*. (1983) and Phillippo *et al* (1987a) studies the reduction in growth rate was accompanied by a decrease in feed intake, which was not affected in the current study. The antagonist effect of Mo and S on feed intake has been proposed to be related to the absorption of thiomolybdates, which may have a direct effect on Cu-dependent enzymes such as peptidylglycine α-amidating monooxygenase that exert its influence on the cholecytokinin and gastrin hormones, regulating appetite (Suttle, 2010).

In the current study, there was no clinical symptoms caused by additional Mo and S. These findings confirm those of Wentink *et al.*, (1999). Knowles *et al.* (2000) reported that there were no clinical signs when dietary Mo increased from 2.2 to 11.7 mg/kg DM in sheep grazing pasture which contained 7.8 mg Cu/kg DM over a period 224 days. Similarly, Williams (2004) reported that dietary Mo at 5 or 10 mg/kg DM produced no clinical symptoms in growing lambs. In contrast, Humphries *et al*. (1983) and Phillippo *et al* (1987a; 1987b) reported clinical signs of Cu deficiency in calves such as a change in colour, infertility, skeletal lesions, reduced growth developed after 20 weeks, when liver Cu dramatically reduced to (3-5 mg/kg DM). Therefore, it is suggested that clinical symptoms

of Cu deficiency as a result of addition Mo can occur after prolonged or severe Cu deficiency and cattle may be more sensitive to Mo than sheep

The mean liver Cu concentration by the end of this study was 292 mg/kg DM in the control group and 190 mg/kg DM in the added Mo and S group, which are well above deficiency limits (15 mg/kg DM) (Sivertsen and Plassen, 2004; Suttle, 2010). In the current study liver Cu concentration and retention was higher when DGP was fed compared with MS, which may be partially related to the higher Cu concentration in the DGP, which in turn, resulted in increased Cu intake by (1.4 mg/d) in lambs (Table 3.8). In general, the availability of Cu in dried feed was found to be higher compared with silage (Suttle, 1980a). Suttle (1980a) demonstrated that Cu absorption was greater in grass hay (5.2-7.2 %) or dried grass (3.1-4.9%) compared with the maize silage (0.9-1.2%).

Petit and Tremblay (1992) demonstrated that the protein degradability of silage, incubated in the rumen using nylon bags, was higher compared with hay potentially due to the extensive proteolysis that occurs in silage during wilting and ensiling processes. The lower Cu availability in fresh forages compared to hay has been attributed to the higher ruminal degradability of dietary protein from fresh forages that contributes to produce higher sulfide, which in turn, reduces Cu availability by the formation of insoluble Cu-sulfide complexes (Ivan, 1993). Therefore, the possibility of less sulfide production in DGP fed lambs due to their lower protein degradability may have contributed to an increase availability of Cu for absorption by animals. An alternative hypothesis may be related to the lower rumen pH in the DGP fed lambs than the MS fed, which in turn, may result in increased sulfide absorption and reduced potency Cu-sulfide interaction (Suttle, 1991). Crosby *et al.* (2004) found that lambs bedded on straw had a lower (15%) liver Cu concentration compared with those housed on an expanded metal floor, which had only access to the concentrate. Crosby *et al.* (2004) suggested that possibly the roughage intake of the straw group would have elevated rumination and salivation and hence increased rumen pH, which in turn, promoted the rumen sulfide producing ciliate protozoa and produced more sulfide. The absorption of up to 50% more dietary Cu (Ivan *et al.*, 1985) and the incidence of chronic Cu toxicity (Dayrell *et al.*, 1994) in fauna free sheep shows the critical role of rumen microflora and sulfide they produce in reducing the availability of dietary Cu.

In the current study, supplementation of Mo and S into the diets resulted in a reduction in liver Cu concentration by approximately 35%, which is lower than the 50% as predicted

using the equation proposed by Suttle and MacLauchlan (1976). However, Crosby *et al.* (2004) also reported a reduction of 37% in the liver Cu concentration by the addition 4 mg/kg DM of Mo to Texel cross lambs. The reduction of liver Cu by the addition of Mo and S was probably due to the formation of insoluble Cu-thiomolybdate complexes in the rumen (Suttle, 1991), which would not be available for absorption, thereby liver Cu concentration decreased in order to meet tissue demand (Robinson *et al*., 1987; Williams, 2004; Suttle, 2012).

Liver Cu status has also been suggested to be depleted by addition of antagonists as a result of a systemic effect of absorbed thiomolybdate, which potentially reduces liver Cu concentration either directly by sequestering Cu from hepatocytes and increasing Cu excretion (Gooneratne, 2012), or indirectly where absorbed thiomolybdate in the blood stream may bind with Cu-albumin and forming an excretable Cu-TM-albumin complex, which is slowly hydrolysised (Manson, 1986), serving as a pool of slowly released Cu, thus resulting in a delay in the transport of Cu to tissues such as the liver (Goonerante *et al*., 1989a; Suttle, 1991; 2010). In addition, absorbed thiomolybdate may bind with Cu with such strong affinity that Cu in Cp is not recycled back to the liver and broken down, and as such the half-life (2-3 d; Linder, 1991) of Cp may be altered, resulting in a reduction in liver Cu concentration (Williams, 2004). The increase in Pl-Cu concentration as a result of the addition of antagonists has been generally attributed to the presence of thiomolybdate in the blood stream (Williams, 2004; Robinson *et al*., 1987). In the current study, blood parameters such as Pl-Cu concentration, Cp activity, and Cp:Pl-Cu ratio were not affected by antagonists. Suttle (2012) also reported no effect of additional 2 mg/kg DM of Mo and 3 g/kg DM of S on Pl-Cu in Texel ram lambs over a period of 96 days. The mean of Pl-Cu concentration was 16 µmol/L, which is higher than the 8-9.4 µmol/L recognised as being marginal for Cu deficiency (Paynter, 1987; Kendall *et al.*, 2000; Suttle, 2010). Therefore, the adverse effect of additional Mo and S on liver Cu status was possibly caused by the direct effect of rumen thiomolybdate on Cu absorption rather than systemic effect of absorbed thiomolybdate.

The form of stored Fe in the body is ferritin and it is principally concentrated in the liver with the normal concentrations in sheep ranging between 70 to 1000 mg/kg DM (Suttle, 2010). The liver Fe concentration in the present study were within normal range although liver Fe status was higher in DGP fed lambs than MS fed lambs. This effect may be due to the higher dietary Fe concentration of DGP which was approximately 2 times higher compared with MS (435 and 248 mg/d respectively), leading to higher Fe intake by lambs. Recently,

Sefdeen *et al* (2014; 2016) showed that increasing Fe concentration in lambs resulted in increased liver Fe concentration.

The SOD activity (Cu-containing enzyme that is involved in preventing the destruction of membrane and intracellular structures against free radicals (Suttle, 2010), was not affected by dietary treatment in the present study. In addition, the mean Hct, Hb, RBC, and WBC were 30.1%, 11.61 g/dL, 11.28 10^6/mm^3, and 10.77 10^3/mm^3, and all values were within the normal range (Jackson and Cockcroft, 2008). The effect of forage type on Hb and RBC counts were observed only at week 4, when DGP fed lambs had higher values for Hb and RBC compared with maize silage fed lambs. This may be due to the higher Fe concentration in DGP, as Fe is essential for Hb formation (Dean, 2005). However, the life span of RBC is estimated to be 120 days (Dean, 2005), which is longer than ten week trial period. Additional antagonists had no effect on haematology parameters, except on one occasion at week 4, when additional antagonists reduced Hct and RBC. The reason for this effect was not clear. Ceruloplasmin is also known as ferroxidase, which is necessary for the oxidation of Fe from ferrous (Fe^{+2}) to ferric (Fe^{+3}) and enables Fe to be mobilised and transported in the blood stream in order to take part in Hb formation, and a reduction in Cp activity may induce anaemia (Suttle, 2010; Prohaska, 2006). As discussed above Cp activity and Pl-Fe concentration were not affected by dietary treatment. It has been reported that additional Mo and S produced no effect on blood haematology parameters in sheep (Williams, 2004). In ruminants, low Hb and Hct is often associated with anaemia caused by prolonged or severe Cu deficiency (Goonerante *et al.*, 1989a; Suttle, 2010).

3.5. Conclusions

The inclusion of Mo and S had no effect on the lamb performance or rumen pH. However, lambs fed DGP had a lower liveweight gain and rumen pH compared with those fed MS. The higher liver Cu status in the DGP fed lambs compared with the MS may be partially related to the higher Cu intake, or lower rumen pH that potentially contributed to an increase in sulfide absorption and decrease Cu-S interaction, and hence increase Cu availability in DGP. The dietary Cu concentration (9 mg/kg DM in DGP and 7 mg/kg DM in MS) resulted in an increased liver Cu concentration in the lambs on DGP diets supplemented or unsupplemented with antagonists and MS unsupplemented with antagonists, except lambs on MS diet supplemented with antagonists, which decreased liver Cu. However, additional antagonists substantially reduced liver Cu status and there was no forage type x antagonist interaction on liver Cu status. Dietary treatment had a small effect on plasma Cu concentration, and Cu-containing enzymes such as ceruloplasmin activity and SOD and it can be concluded that using these parameters to determine Cu status is insensitive.

To conclude, there was no interaction between forage type and antagonists on Cu status throughout the study. The reasons for higher liver Cu status in the DGP fed lambs compared with MS may possibily be related to the lower rumen pH in DGP. Reasons for the rumen pH effect was in the current study was not clear and require further investigation. Using DGP to simulate the differences in Cu metabolism between dried vs. silage forages may not be ideal due to the smaller feed particle size with the consequence of a high out flow rate from the rumen.

Chapter 4 The effects of forage type and inclusion of molybdenum and sulfur supplementation on copper status in growing lambs

4.1. Introduction

The availability of Cu is different between feedstuffs consumed by ruminants and Cu is well absorbed from non-fibrous feedstuffs such as cereals compared with fresh or conserved forages. Suttle (2012) demonstrated that liver Cu concentration in Texel lambs reached values associated with Cu toxicity (1069 mg/kg DM) by feeding a concentrate diet containing 90.8% of whole barley grain. Conservation of grass as hay or silage generally improves Cu availability compared to fresh grass (Suttle, 1983b; 1986; 2010). This was confirmed by Suttle (1983b) who reported that Cu absorption in sheep was higher in hay compared with fresh herbage. In Chapter 3, dried grass pellets resulted in a higher liver Cu status than maize silage. In addition, the inclusion of Mo and S was found to lower liver Cu status, although no interaction between forage type and antagonists was observed. The degree of thiomolybdate produced in the rumen is due to interactions occurred between molybdenum and sulfur and has been reported to be affected by forage type, however, the reason for this effect is not clear (Suttle, 1980a; 1986; 2010). For instance, the inhibition effect of Mo on Cu absorption in grass hays is less than that of S, while in fresh grass a small increment of Mo and S greatly reduced Cu absorption, with semi-purified diet being intermediate (Suttle, 1983b). More recently, Sinclair *et al*. (2017) demonstrated that the liver Cu status in cows fed maize silage was higher compared with those fed grass silage, and the extent of reduction of liver Cu status caused by antagonists was more pronounced when grass silage was fed compared with maize silage, although the reason for these effects were not clear. Therefore, this would suggest that the forage type is an essential factor that should be taking in to account in calculating Cu requirements for ruminants, especially when dietary Mo and S are high.

In Chapter 3, Texel cross breed lambs were used to investigate effects of diet on Cu metabolism. Breeds of sheep vary in Cu absorption and those that absorb Cu well such as Texel, North Ronaldsay and Suffolk breeds are more prone to Cu toxicity than deficiency (Suttle *et al*., 2012), while breeds such as Scottish Blackface or Welsh Mountain are relatively susceptible to Cu deficiency (Woolliams *et al*., 1986a;1986b). Liver Cu accumulation, which reflects absorptive efficiency, was found to be higher in the Texel breed compared with other breeds (Woolliams *et al*., 1983; Suttle *et al*., 2002). Moreover, as shown in Chapter 3 dried grass pellets resulted in a lower rumen pH than maize silage potentially due to having smaller feed particle size, however, dried forages have been recognised to have a higher rumen pH due to their buffering capacity and provision of more

saliva compared with silages such as maize that contains high level of easily digested carbohydrates, which depresses rumen pH (Ørskov, 1987; MacDonald, *et al*., 2011). Therefore, the aims of this study were to further evaluate the effects of forage type and addition of Mo and S on Cu metabolism in growing lambs using breeds more prone to Cu deficiency.

4.2. Materials and methods

4.2.1. Animal procedures

All procedure involving animals were carried out according to the UK Animals (Scientific Procedures) Act 1986 and were approved by the Harper Adams University Ethic Committee.

4.2.2. Animals and experimental design

The study was carried out at Harper Adams University (at 3rd of November 2014) using 48 castrated male Swaledale lambs with an initial mean body weight of 27.1 kg (s.e.d; 0.35) over a period of 10 weeks. Eight representative lambs were slaughtered immediately prior to the start of the study in a commercial abattoir, and liver samples were collected and stored at -20 °C prior to serve as a baseline for liver Cu levels. The remaining 40 lambs were blocked according to liveweight (LW) and then randomly allocated to one of four treatments in a 2 x 2 factorial design with 10 lambs per treatment. The lambs were housed in a well-ventilated shed in individual pens and bedded on wood shavings. They had free accessed to water.

4.2.3. Diets

Lambs were fed diets with a forage to concentrate ratio of 60:40 (DM basis) to meet their requirements to grow at 200 g/day (AFRC, 1993). The forages were either grass haylage (GH) or maize silage (MS). Appropriate concentrates were formulated to obtain an isonitrogenous, isoenergetic diet (Table 4.1). The predicted metabolisable energy (ME) of the experimental diets was 11.56 and 11.65 (MJ/kg DM) for GH and MS respectively (AFRC, 1993). The GH was from a perennial ryegrass mix sward, second cut, and harvested at Harper Adams University in the middle of July 2015. The MS forage was also made at Harper Adams University.

Table 4.1. Raw material composition of the experimental concentrates (g/kg DM).

Ingredients, g/kg DM	Concentrate Diets[1]	
	GH	MS
Barley	352	317
Sugar beet pulp	102	128
Soya bean meal	315	412
Molasses	77	77
Megalac	86	---
Urea[2]	11	9
Mins/vits[3]	57	57
Total	1000	1000

[1] GH= concentrate fed with grass haylage forage, MS= concentrate fed with maize silage.

[2] Urea (Trouw Nutrition, Cheshire, UK).

[3] Mineral premix (25 kg/tonne) (Rumenco, Burton upon Trent, Staffordshire, UK). Major minerals (g/kg DM): Calcium, 185; Phosphorous, 20; Magnesium, 100; Sodium, 120; Chloride, 205; Trace elements (mg/kg DM); Iodine, 150; Cobalt, 90; Manganese, 3000; Zinc, 3000; Selenium (sodium selenite), 20. Vitamins; Vit A {E 672}, 320000 IU/kg; Vit D3 {E 671}, 100000 IU/kg. Vit E (all-rac-alpha-tocopheryl acetate) {3a700} 2000 mg/kg.

The individual components of the diet were analysed by ICP-MS (section 2.4.1) (Table 4.2) and trace element supply (Table 4.2) was calculated. Based on the equations of Suttle and MacLauchlan (1976) Mo and S was added to the diets to reduced Cu absorption by 50%. Levels added are presented in Table 4.3. The Mo added was in the form ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Fisher Scientific, Leicester, UK), and S wan in the form ammonium sulphate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA). The N content of the diets were balanced with feed grade urea (Trouw Nutrition, Cheshire, UK).

Table 4.2. Chemical composition of grass haylage and maize silage

Item	Grass haylage	Maize silage
Chemical composition, g/kg DM		
DM, g/kg	868	324
CP,	96	73
NDF,	654	381
EE,	11	34
Ash,	63	37
ME, ML/kg DM[1]	10.0	11.30
Mineral composition, mg/kg DM		
Cu,	6.3	4.7
Mo,	1.4	0.5
S, g/kg DM	1.4	0.9
Fe,	151.2	82.8
Zn,	22.6	20.6
Mn,	125.7	14.5

ME= metabolisable energy was taken from AFRC (1993)

Table 4.3. The predicted mineral composition for the experimental diets.

	Grass haylage		Maize silage	
	No added Mo and S	Added Mo and S	No added Mo and S	Added Mo and S
Cu, mg/kg DM	10.8	--	10.5	--
Mo, mg/kg DM	2.02	3.5	1.81	3.5
S, g/kg DM	2.42	2	2.28	2.2

Therefore, lambs were allocated according to their liveweight to one of four dietary treatments (Table 4.4).

Table 4.4. Dietary treatments

Code	Treatments
GH-	0.60 grass haylage: 0.40 concentrate (DM basis), no addition of antagonists
GH+	0.60 grass haylage: 0.40 concentrate (DM basis), with additional Mo and S
MS-	0.60 maize silage: 0.40 concentrate (DM basis), no addition of antagonists
MS+	0.60 maize silage: 0.40 concentrate (DM basis), with additional Mo and S

Feed samples (forage and concentrates) were collected weekly throughout the study. At the end of the study, all feed samples were analysed for DM, Ash, CP, NDF, EE and mineral contents as described in sections 2.1.1. to 2.1.5, and section 2.4.1 respectively. The chemical composition of the experimental diets are presented in Table 4.5.

Table 4.5. Analysed chemical and mineral composition of the experimental diets supplying 600 g/kg DM forage and 400 g/kg DM concentrates (60:40)[1].

	GH-	GH+	MS-	MS+
Chemical composition, g/kg DM				
DM, g/kg	859.1	860.2	530.7	532.7
CP,	156.0	158.1	163.9	165.8
NDF,	450.6	452.8	290.7	292.3
EE,	31.4	32.2	62.8	64.0
Ash,	82.6	86.7	62.5	62.4
Mineral composition, mg/kg DM				
Cu,	11.0	11.6	10.7	10.7
Mo,	1.8	4.5	1.5	4.2
S, g/kg DM	1.9	3.3	1.8	3.2
Fe,	179.8	179.0	151.5	151.7
Zn,	71.6	69.8	67.9	69.9
Mn,	119.0	120.8	53.7	51.3

[1] Diets consists of either grass haylage (GH) + concentrate or maize silage (MS) + concentrate at a ratio of 60:40 forage: concentrate. Diets DGP+ or MS+ received additional Mo and S, resulting in a Mo content of 5 mg/kg DM and S content of 4 g/kg DM.

4.2.4. Experimental routine

All lambs were offered feed twice a day at (08:30 and 16:30h). The GH forage was chopped (approximately 5 cm length) before being fed to the lambs using a straw chopper (New Wic Bedding-Straw Chopper, Lancashire, UK). Forages (GH and MS) were put into wooden troughs, and concentrates placed into plastic buckets. Feed refusals were collected twice a week (every Monday and Thursday until the end of experiment) to estimate individual feed intake and feed conversion efficiency. The quantity of the feed offered was adjusted weekly according to the live weight of the animal taken on the day of live weight determination (section 4.2.4.2) to meet AFRC (1993) requirement. At the end of the study, lambs were sent to a commercial abattoir for slaughter. All lambs, including the eight representative lambs slaughtered on day 0, were slaughtered following electrical stunning. Livers were collected immediately after slaughtering, weighed, and stored at -20°C for subsequent mineral content determination.

4.2.4.1. Blood sample collection and analysis

Blood samples were collected by jugular vein puncture (section 2.2.) once a week on Wednesday at 11:00h for plasma and serum analysis (sections 4.2.5). On weeks 0, 4, 8, and 10 an additional EDTA tube was collected for haematology analysis and an aliquot stored at -20°C for SOD analysis (section 4.2.5). Blood samples (week 0) were collected on 3rd of June 2014.

4.2.4.2. Liveweight determination

Lambs were weighed once a week on Thursday at 11:00 using a standard operating procedure as described in section 2.3. Daily liveweight gain (DLWG) was calculated using regression analysis.

4.2.5. Blood analysis

Fresh blood samples after being collected were directly analysed for haematocrit (Hct), haemoglobin concentration (Hb), red blood cell counts (RBC), and while blood cell counts (WBC) using a Vet Animal Blood Counter (section 2.2.1). Frozen samples of whole blood, plasma, and serum were defrosted thoroughly at room temperature. Whole blood samples were analysed for SOD activity using a Cobas Mira Plus analyser as described in section 2.2.3.1. Plasma samples were used to determine mineral concentration (section 2.2.2), and trichloroacetic acid soluble concentration (section 2.2.2.1). Blood serum samples were also analysed for ceruloplasmin activity (Cp) using a Cobas Mira Plus (section 2.2.3.2).

4.2.6. Liver mineral concentrations

Liver samples were analysed for mineral concentrations using an ICP-MS as described in section 2.4.2. Whole liver mineral content was determined by multiplying liver mineral concentrations by liver weight and by liver DM. Liver mineral retention was determined by subtracting whole liver mineral content of the initial slaughter group from final whole liver minerals content, divided by days of the study period.

4.2.7. Rumen pH determination

Rumen fluid samples were collected immediately after slaughter of the lambs, put into 100 ml plastic pots and stored on ice prior to measuring pH within an hour after slaughtering (section 2.1.6).

4.2.8. Statistical analysis

Performance, plasma minerals, haematology, and enzyme activities were analysed by repeated-measures ANOVA as a 2x2 factorial randomise block design with the main effects of forage type (F), antagonists (Ant.), and interaction between forage type and antagonists (Int.). Daily live weight gain (DLWG) was calculated by regression analysis and analysed by ANOVA. For plasma Mn concentration and Cp:Pl-Cu ratio week zero was used as a covariate. All statistical analysis were conducted using Genstat version 17.1 (Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Significance was set at $P < 0.05$ and trends at $P < 0.10$. Significant differences between means were tested using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

4.3. Results

4.3.1. Health observation

Additional Mo and S in the current study did not produce clinical symptoms, and all lambs were healthy and none were not removed from the study.

4.3.2. Animal performance and intake

There was no forage x antagonist interaction (P>0.05) on weekly liveweight (Table 4.7), DLWG, DMI, and FCE of the lambs throughout the study (Table 4.6). There was also no effect (P>0.05) of antagonists on these parameters. However, lambs fed the maize silage were heavier (P<0.05) from week 6 until the end of the study compared with lambs fed the grass haylage (Fig. 4.1). The grass haylage fed lambs had a higher (P<0.05) forage and total DMI compared with the maize silage fed lambs, although concentrate DMI was not different (P>0.05) between treatments. The DLWG and FCE were higher (P<0.05) in the maize silage fed lambs compared with the grass haylage fed lambs.

Table 4.6. Intake, performance, and rumen pH of growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented either without (-) or with (+) Mo and S.

Items	Treatment				Significance[1]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Intake, kg/d								
Forage DMI,	0.48	0.50	0.41	0.45	0.033	0.020	0.197	0.845
Concentrate DMI,	0.34	0.34	0.34	0.33	0.010	0.321	0.483	0.619
Total DMI	0.82	0.85	0.75	0.78	0.041	0.029	0.398	0.992
DLWG, kg/d	0.07	0.08	0.13	0.13	0.017	<.001	0.925	0.708
FCE[2]	0.08	0.08	0.18	0.16	0.016	<.001	0.541	0.522

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

[2] FCE calculated as DLWG (kg/d) divided by DMI (kg/d).

Table 4.7. Liveweight in growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Week	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	27.3	27.3	27.0	27.0	0.35	0.276	0.920	0.920
1	29.8	28.7	28.1	29.0	1.02	0.358	0.918	0.188
2	29.5	29.4	29.0	29.7	0.72	0.846	0.560	0.497
3	30.1	29.9	29.7	30.3	0.80	0.93	0.726	0.485
4	30.3	30.5	30.9	31.3	1.09	0.373	0.654	0.898
5	31.2	31.2	32.0	32.4	1.00	0.180	0.753	0.807
6	31.2	31.2	32.7	33.2	0.95	0.016	0.686	0.741
7	31.8	31.6	33.5	34.0	1.01	0.008	0.780	0.626
8	32.5	32.6	34.5	35.0	1.12	0.008	0.708	0.803
9	32.6	32.3	35.5	35.4	1.20	0.001	0.838	0.884
10	32.8	32.6	36.7	36.6	1.30	<.001	0.892	0.978

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

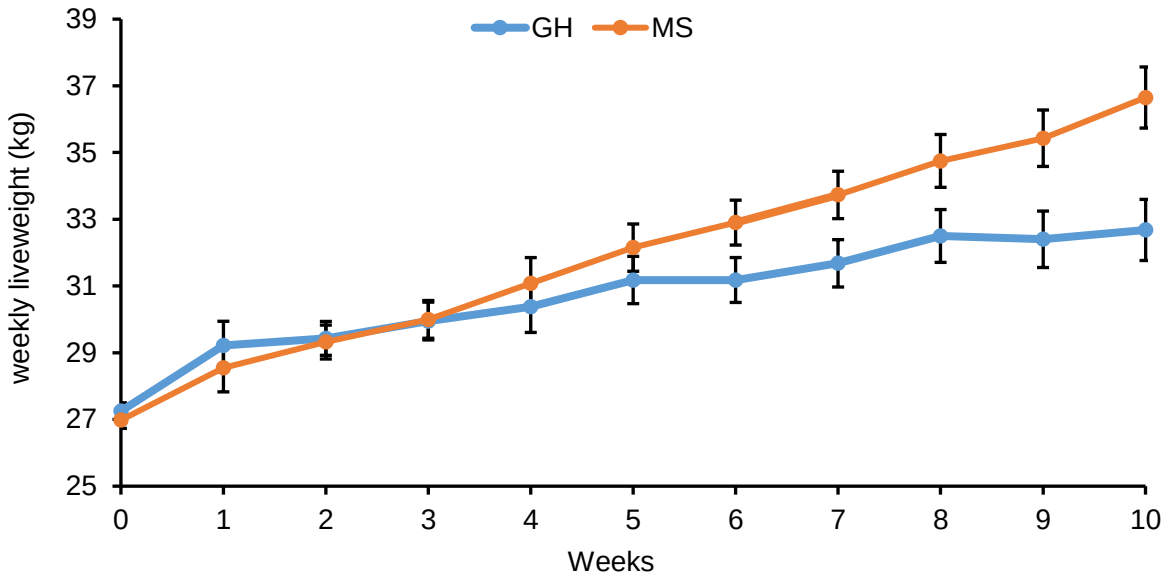


Figure 4.1. Effect of forage type grass haylage (GH) or maize silage (MS) on weekly liveweight in growing lambs. Error bars indicate s.e.d.

4.3.3. Mineral intake

There was no forage x antagonist interaction (*P*>0.05) on Cu, S, Fe, Zn, and Mn intake (Table 4.8). However, there was forage x antagonist interaction (*P*<0.05) on Mo intake, which was higher in lambs fed grass haylage or maize silage supplemented with antagonists, intermediate in lambs fed grass haylage no added antagonists and lowest in lambs fed maize silage unsupplemented with antagonists, Additional antagonists resulted in an increased (*P*<0.001) in S intake in comparison with the lambs not receiving antagonists. There was no difference in Mo intake between lambs offered grass haylage or maize silage (*P*>0.05). Lambs offered grass haylage had a higher (*P*<0.05) Cu, S, Fe, Zn, and Mn intake compared with the lambs offered maize silage.

Table 4.8. Mineral intake in growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Minerals, mg/d	Treatment				Significance			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int
Cu,	9.0	9.8	8.1	8.3	0.50	<.001	0.111	0.404
Mo,	1.5[b]	3.1[c]	1.2[a]	3.3[c]	0.13	0.358	<.001	0.009
S, g/d	1.6	2.8	1.3	2.5	0.11	0.004	<.001	0.805
Fe,	147.6	151.4	114.2	118.1	6.73	<.001	0.424	0.986
Zn,	58.8	59.0	51.2	54.4	2.87	0.006	0.407	0.456
Mn,	97.7	102.1	40.5	40.0	3.67	<.001	0.455	0.351

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

4.3.4. Rumen pH

There was no forage x antagonist interaction (*P*>0.05) on rumen pH. There was also no effect (*P*>0.05) of the antagonists on rumen pH (Table 4.9). However, lambs on maize silage had a lower rumen pH compared with those on grass haylage (*P*<0.001) (Fig. 4.2).

Table 4.9. Rumen pH of growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented either without (-) or with (+) Mo and S.

	Treatment					Significance[1]		
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Rumen pH	6.19	6.25	6.00	5.84	0.094	<.001	0.470	0.113

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

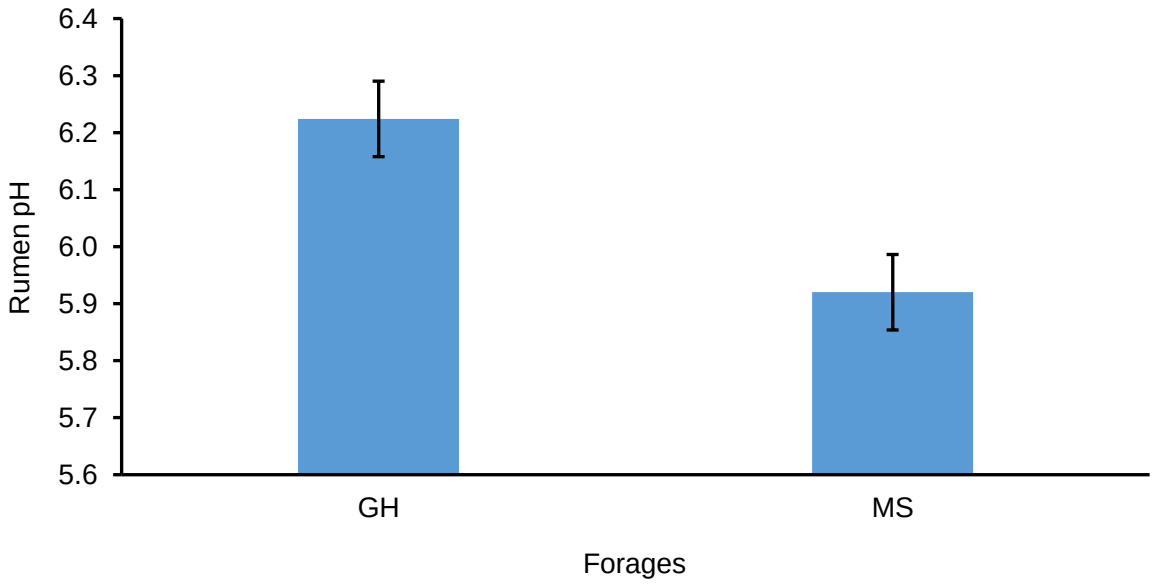


Figure 4.2. Rumen pH of growing lambs fed diets based on grass haylage (GH) or maize silage (MS). Error bars indicate SED.

4.3.5. Liver mineral status

4.3.5.1. Liver mineral concentration

The mean liver concentration of Cu, Mo, Fe, Zn and Mn of the lambs slaughtered at the beginning of the experiment is present in Table 4.10

There was no forage x antagonist interaction on liver Cu concentration (P>0.05) (Table 4.11). Likewise, there was no effect (P>0.05) of forage type on liver Cu concentration. Lambs fed diets supplemented with Mo and S had a lower (P<0.05) (41.3%) liver Cu concentration compared with those fed unsupplemented diets, with mean values of 131 and 223 mg/kg DM (s.e.d; 36.9) respectively.

Liver Mo concentration was higher (P<0.05) in lambs on grass haylage compared with those on maize silage, with mean values of 5.1 and 4.6 mg/kg DM (s.e.d; 0.20) respectively. There was no effect (P>0.05) of dietary treatment on liver Fe, Zn, and Mn concentrations (P>0.05).

Table 4.10. Liver mineral concentrations of (8) representative lambs slaughtering at the starting of the study.

Liver minerals, mg/kg DM	Concentration (mg/kg DM)	Standard Deviation
Cu	268.4	± 132.3
Mo	4.9	± 0.5
Fe	416.2	± 82.7
Zn	217.5	± 71.0
Mn	31.1	± 10.5

Table 4.11. Liver mineral concentrations of growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added Mo and S.

Minerals, mg/kg DM	Treatments				Significance			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Cu,	192	125	255	138	52.2	0.308	0.019	0.499
Mo,	5.5	5.2	4.7	4.5	0.28	0.018	0.937	0.301
Fe,	508	484	580	348	171.7	0.792	0.300	0.399
Zn,	136.8	132.9	123.9	126.3	7.71	0.085	0.895	0.565
Mn,	47.1	30.1	34.9	32.9	7.56	0.391	0.086	0.171

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

4.3.5.2. Whole liver mineral content

There was no forage x antagonist interaction (*P*>0.05) on whole liver content of all minerals (Table 4.12). There was also no effect (*P*>0.05) of antagonists on whole liver content of Mo, Zn, Fe, and Mn. However, whole liver Cu content was lower in lambs supplemented with Mo and S compared with the lambs not receiving antagonists (*P*<0.05). Forage type had no effect (*P*>0.05) on whole liver Fe and Mn content. Lambs fed maize silage had a higher whole liver Cu, Mo, and Zn content compared with the lambs fed grass haylage (*P*<0.05).

Table 4.12. Whole liver minerals content[1] of growing lambs fed diets based on forages grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S[1].

Minerals, mg/liver	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Cu,	19.5	11.5	32.9	17.0	5.76	0.028	0.007	0.337
Mo,	0.51	0.50	0.60	0.62	0.071	0.039	0.934	0.788
Fe,	50.0	47.6	72.5	45.7	20.39	0.482	0.320	0.408
Zn,	13.8	12.9	15.9	16.9	1.65	0.013	0.990	0.418
Mn,	4.7	2.9	4.5	4.3	0.83	0.310	0.097	0.198

[1] whole liver mineral content = final whole liver weight x final liver DM x final liver Cu concentration (mg/kg DM).

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

4.3.5.3. Whole liver mineral retention

There was no forage x antagonist interaction on whole liver retention (*P*>0.05) for all minerals (Table 4.13). Compared to the total liver Cu content in the initial slaughter group, lambs fed the grass haylage had a lower whole liver Cu retention compared with those fed maize silage diet, with mean values of -0.12 and 0.02 mg/d respectively. Lambs fed diets unsupplemented with antagonists had a higher whole liver Cu retention than those fed diets supplemented with antagonists, with mean values of 0.03 and -0.14 mg/d respectively. There was no effect of antagonists on whole liver retention of Mo, Fe, Zn and Mn (*P*>0.05). There was no effect of forage type on whole liver Fe and Mn retention (*P*>0.05). However, there was an effect (*P*<0.05) of forage type on liver Mo, and Zn retention, which was higher (*P*<0.05) in lambs fed maize silage compared with those fed grass haylage.

Table 4.13. Liver minerals retention[1] of growing lambs fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S.

Minerals, mg/d	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Cu,	-0.06	-0.18	0.13	-0.10	0.082	0.028	0.007	0.337
Mo, µg/d	1.09	0.96	2.45	2.70	1.008	0.039	0.934	0.788
Fe,	0.20	0.17	0.53	0.14	0.291	0.482	0.320	0.408
Zn,	-0.07	-0.09	-0.04	-0.03	0.024	0.013	0.990	0.418
Mn, µg/d	27.9	2.4	25.6	22.2	11.88	0.310	0.097	0.198

[1] liver minerals retention were calculated by substracting whole liver mineral content at day zero from final whole liver Cu content divided by whole study period (days).

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference

4.3.6. The mean of plasma mineral profile, Cu-mediated enzymes, and haematology profile

There was no forage type x antagonist interaction (*P*>0.05) on the mean Pl-Cu concentration (Table 4.14). There was also no effect (*P*>0.05) of forage type on the mean Pl-Cu concentration. However, the mean Pl-Cu concentration was higher in lambs fed diets unsupplemeted with antagonists compared with those fed diets supplemented with antagonists. There was a forage x antagonist interaction (*P*<0.001) on the mean Pl-Mo concentration, where the addition of antagonists in lambs fed grass haylage resulted in an increase (*P*<0.05) in the mean Pl-Mo concentration compared with the lambs fed grass haylage unsupplemented with antagonists. However, the addition of antagonists had no effect (*P*>0.05) on the mean Pl-Mo concentration in lambs fed the maize silage diet. The mean Pl-Fe and Zn concentrations were not affected by dietary treatment (*P*>0.05).

There was no effect of forage x antagonist interaction, or forage type on the mean Cp activity. Lambs fed diets supplemeted with Mo and S had a lower (*P*<0.05) mean Cp activity compared with thosed fed diets unsupplemented with antagonists. No effect of dietary treatment was observed on the mean Cp:Pl-Cu ratio (*P*>0.05). The mean SOD activity also was not affected by dietary treatment (*P*>0.05). No effect was observed of dietary treatment on the mean of Hct (%), Hb concentration, or WBC counts (*P*>0.05). Lambs fed grass haylage has a higher RBC count compared to those fed maize silage (*P*<0.05).

Table 4.14. Effect of forage type grass haylage (GH) and maize silage (MS) fed without (-) or with (+) added Mo and S on mean indicators of blood Cu status over the study period of lambs[1].

Items	Treatments				Significance			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
Cu, µmol/L	15.1	13.5	15.1	12.8	1.542	0.637	0.015	0.673
Mo, µmol/L	0.16	0.41	0.21	0.19	0.040	<.001	<.001	<.001
Fe, µmol/L	46.0	44.7	46.6	46.6	6.11	0.572	0.790	0.765
Zn, µmol/L	9.3	9.4	9.6	8.9	0.8628	0.799	0.321	0.143
Cp, mg/dL	10.9	8.7	10.6	9.1	1.48	0.937	0.012	0.575
Cp:Pl-Cu	0.73	0.66	0.70	0.68	0.075	0.999	0.058	0.310
SOD, U/g of Hb	1836	1891	1880	1763	213.3	0.750	0.811	0.515
Hct, %	37.2	39.2	37.5	37.1	1.31	0.257	0.277	0.126
Hb, g/dL	13.0	13.6	13.0	13.1	0.40	0.296	0.171	0.182
RBC, $10^6/mm^3$	13.5	13.7	12.8	12.7	0.42	0.004	0.969	0.619
WBC,$10^3/mm^3$	9.0	7.9	8.5	9.1	0.66	0.547	0.633	0.143

[1] week 0 values were used as a covariate where appropriate.

[2] SOD= superoxide dismutase activity; Hct- haematocrit; Hb= haemoglobin; RBC= red blood cells; WBC= white blood cells.

[3] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

4.3.7. Plasma mineral concentrations

4.3.7.1. Plasma copper concentration

There was an effect (*P*<0.001) of time on Pl-Cu concentration, with plasma levels declining over the period of the study. However, there was no effect (*P*>0.05) of time x treatment interaction on Pl-Cu concentration.

There was no forage x antagonist interaction (*P*>0.05) on Pl-Cu concentration at any weekly time points (Table 4.15). Similarly, there was no effect of forage type on Pl-Cu concentration (*P*>0.05) at any weekly time points. However, during weeks 3, 8, and 10 lambs fed diets supplemented with Mo and S had a lower (*P*<0.05) Pl-Cu concentration compared with the lambs fed diet no added Mo and S. There was also a trend (*P*<0.1) for supplemented with antagonists lambs to have a lower Pl-Cu on all other dates.

Table 4.15. Plasma copper concentration of growing lambs fed diets based on forages grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

Week	Treatment					Significance[2]		
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	19.1	20.5	19.1	17.3	1.58	0.154	0.862	0.176
1	20.7	19.7	21.4	17.1	2.19	0.539	0.096	0.299
2	17.7	16.8	19.5	15.2	1.97	0.934	0.071	0.226
3	15.5	13.6	15.6	13.6	1.29	0.925	0.038	0.916
4	15.3	12.4	14.6	13.0	1.65	0.925	0.065	0.571
5	14.0	11.9	14.2	12.6	1.35	0.657	0.060	0.773
6	13.0	11.3	13.9	11.6	1.51	0.620	0.069	0.778
7	12.1	10.8	12.7	10.6	1.23	0.795	0.069	0.669
8	12.5	10.8	12.4	10.3	0.93	0.648	0.006	0.716
9	13.1	11.0	11.6	9.7	1.44	0.180	0.066	0.912
10	13.5	9.7	11.3	9.8	1.63	0.355	0.029	0.317

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.58	<.001
Time x Forage effect	0.76	0.480
Time x Antagonist effect	0.76	0.553
Time x Forage x Antagonist effect	1.09	0.260

4.3.7.2. Plasma molybdenum concentration

There was an effect (*P*<0.001) of time on Pl-Mo concentration, which increased at week 1 and then fluctuated. There was also a time x treatment interaction on Pl-Mo concentration (*P*<0.05).

There was a forage x antagonist interaction (*P*<0.001) on Pl-Mo concentration from week 2 until week 10 (Table 4.16). Lambs fed grass haylage supplemented with Mo and S had a higher Pl-Mo levels compared with all other groups from week (*P*<0.001). The unsupplemented lambs fed grass haylage had the lowest Pl-Mo concentration on weeks 2, 3, 4, 5, 6, 8, and 10 compared with both groups fed maize silage unsupplemented Mo and S and grass haylage fed lambs supplemented with Mo and S.

Table 4.16. Plasma molybdenum concentration of growing lambs fed diets based on forages grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

Week	Treatment[2]					Significance[3]		
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	0.25	0.20	0.19	0.19	0.046	0.360	0.524	0.502
1	0.29	0.35	0.35	0.25	0.061	0.624	0.608	0.079
2	0.15^{a}	0.56^{c}	0.26^{b}	0.24^{b}	0.041	0.001	<.001	<.001
3	0.11^{a}	0.47^{c}	0.20^{b}	0.20^{b}	0.040	0.004	<.001	<.001
4	0.14^{a}	0.61^{b}	0.20^{a}	0.24^{a}	0.054	<.001	<.001	<.001
5	0.10^{a}	0.43^{c}	0.29^{b}	0.20^{ab}	0.053	0.471	0.003	<.001
6	0.12^{a}	0.44^{c}	0.20^{a}	0.20^{a}	0.027	<.001	<.001	<.001
7	0.22^{a}	0.35^{b}	0.20^{a}	0.17^{a}	0.029	<.001	0.018	<.001
8	0.13^{a}	0.35^{b}	0.15^{a}	0.16^{a}	0.021	<.001	<.001	<.001
9	0.17^{a}	0.44^{b}	0.15^{a}	0.15^{a}	0.032	<.001	<.001	<.001
10	0.10^{a}	0.36^{c}	0.12^{ab}	0.15^{b}	0.026	<.001	<.001	<.001

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] ^{a,b,c} Means within a row with different superscripts are significantly different at (*P*<0.05).

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.018	<.001
Time x Forage effect	0.016	0.007
Time x Antagonist effect	0.016	<.001
Time x Forage x Antagonist effect	0.029	<.001

4.3.7.3. Plasma iron concentration

There was an effect (*P*<0.001) of time on Pl-Fe concentration (Fig. 4.3). However, there was no effect of time x treatment interaction on Pl-Fe concentration (*P*>0.05).

There was no effect (*P*>0.05) of dietary treatment on Pl-Fe concentration at any weekly time

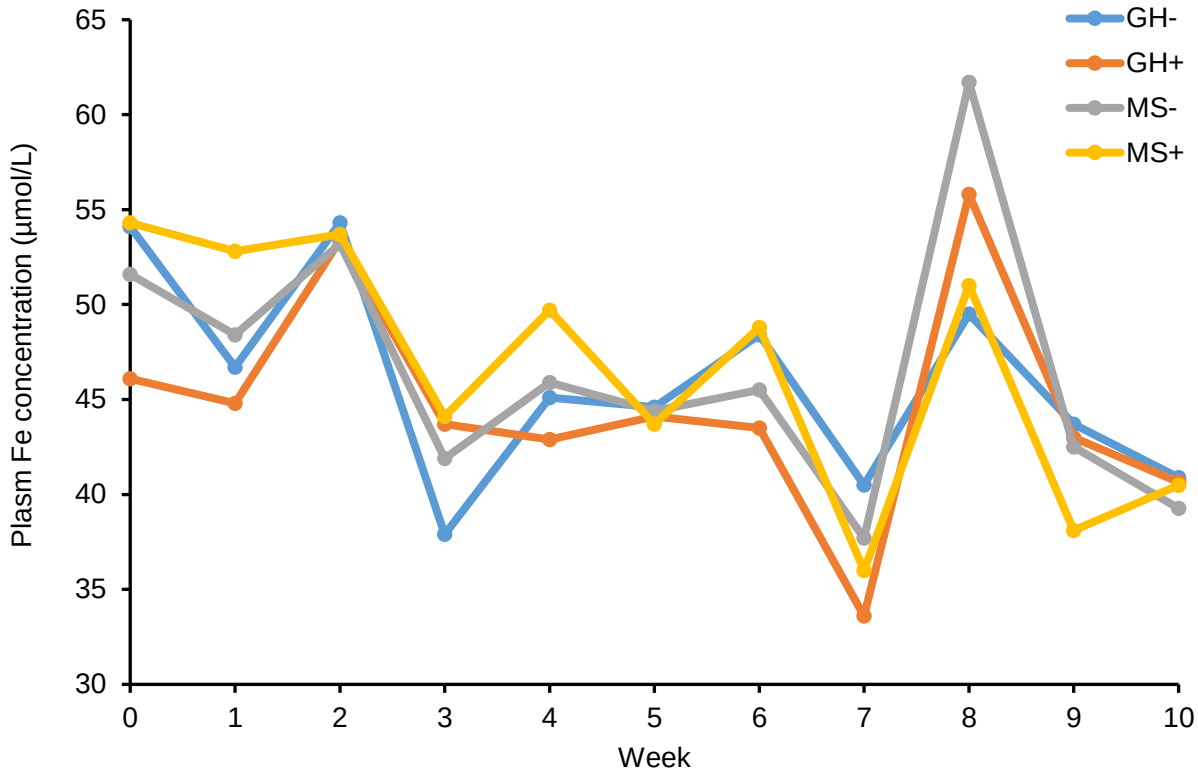


Figure 4.3. Plasma iron concentration of growing lambs fed diet based on grass haylage (GH) and maize silage (MS) supplemented without (-) or with Mo and S (µmol/L). Error bars indicate SED. Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	2.74	<.001
Time x Forage effect	2.24	0.816
Time x Antagonist effect	2.24	0.842
Time x Forage x Antagonist effect	4.32	0.422

4.3.7.4. Plasma zinc concentration

There was an effect (P<0.05) of time on Pl-Zn concentration (Fig. 4.4). There was no effect of time x treatment interaction on Pl-Zn concentration (P>0.05).

There was no effect (P>0.05) of dietary treatment on Pl-Zn concentration throughout the study.

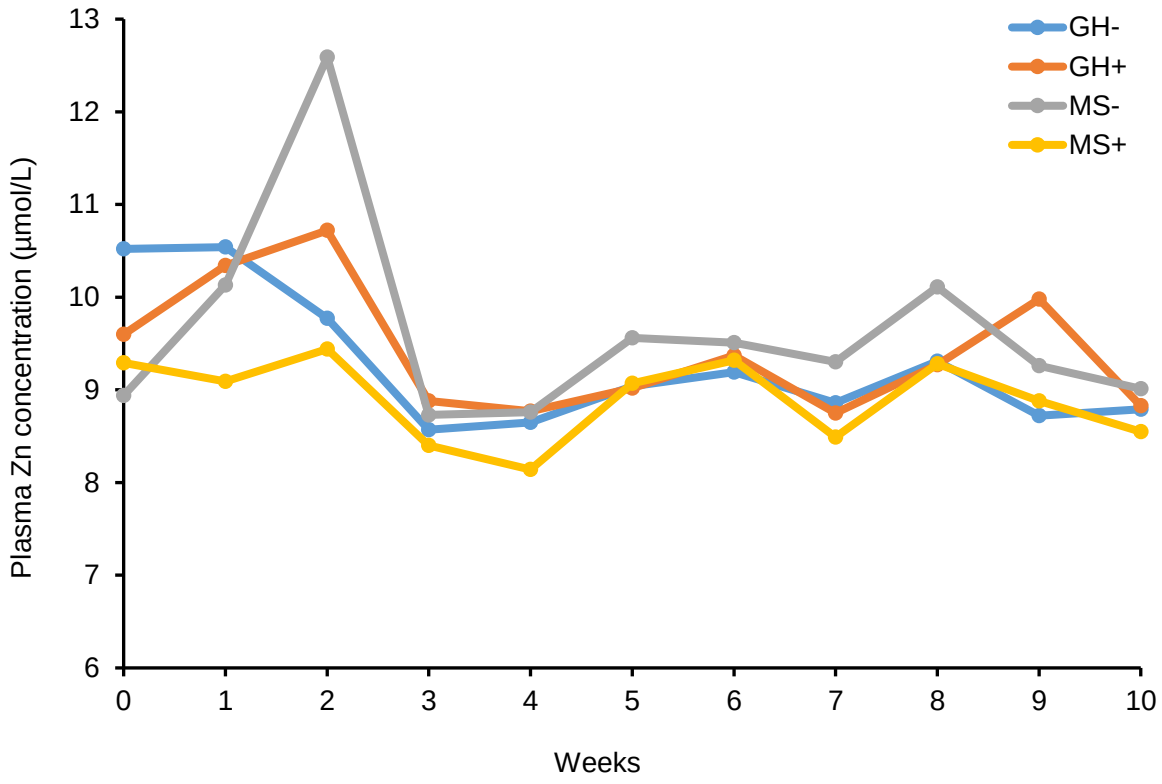


Figure 4.4. Plasma zinc concentration of growing lambs fed diet based on grass haylage (GH) and maize silage (MS) supplemented without (-) or with Mo and S (µmol/L). Error bars indicate SED. Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.40	0.012
Time x Forage effect	0.29	0.444
Time x Antagonist effect	0.29	0.612
Time x Forage x Antagonist effect	0.61	0.283

4.3.8. Ceruloplasmin activity

There was an effect (*P*<0.001) of time on Cp activity, with Cp activity decreasing over a period of the study (Table 4.17). There was no time x treatment interaction (*P*>0.05) on Cp activity.

At week 1, there was an interaction (*P*<0.05) between forage type and antagonists on Cp activity. The highest Cp activity was in lambs fed maize silage unsupplemented with antagonists compared with lambs fed any of the other diets. At week 9, there was also a forage x antagonist interaction (*P*<0.05) on Cp activity. The addition of antagonists reduced Cp activity in lambs fed grass haylage compared with the lambs fed grass haylage diet unsupplemented with antagonists, while there was no difference (*P*>0.05) in Cp activity in lambs fed maize silage diets unsupplemented or supplemented with antagonists. There was no effect (*P*>0.05) of forage type on Cp activity at any weekly time points. However, during week 3, 4, 7 until 9 lambs fed diets supplemented with Mo and S had a lower Cp activity compared with lambs not receiving antagonists (*P*<0.05).

Table 4.17. Ceruloplasmin activity of growing lambs fed diets based on forages grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) added S and Mo and S(mg/dL)[1].

Week	Treatment[2]					Significance[3]		
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	15.4	12.6	13.6	12.1	2.04	0.441	0.149	0.641
1	11.4^{a}	13.2^{ab}	14.4^{b}	11.6^{a}	1.33	0.479	0.559	0.020
2	10.2	9.6	10.4	9.0	1.50	0.867	0.345	0.713
3	11.9	7.9	10.7	8.6	1.21	0.819	0.002	0.289
4	10.6	8.1	10.0	7.5	1.27	0.549	0.010	0.993
5	11.1	7.6	10.7	10.8	1.44	0.178	0.104	0.090
6	11.2	6.5	9.9	7.2	2.61	0.864	0.054	0.602
7	8.5	6.2	8.5	6.8	1.28	0.737	0.035	0.788
8	9.9	8.1	9.9	7.9	1.03	0.895	0.013	0.880
9	10.8^{b}	7.8^{a}	9.9^{b}	9.6^{A}	0.81	0.597	0.016	0.016
10	7.5	6.3	6.1	5.9	1.06	0.251	0.326	0.478

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] ^{a,b} Means within a row with different superscripts are significantly different at (*P*<0.05).

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.60	<.001
Time x Forage effect	0.67	0.507
Time x Antagonist effect	0.67	0.249
Time x Forage x Antagonist effect	1.05	0.141

4.3.9. Ceruloplasmin to plasma copper ratio

There was an effect of time on Cp:Pl-Cu ratio (P<0.001), with Cp:Pl-Cu ratio declining at week two and then fluctuated (Table 4.18). There was a time x antagonist interaction on Cp:Pl-Cu ratio (P<0.05). There was also a trend (P=0.092) for a time x forage type x antagonist interaction on Cp:Pl-Cu ratio. However, there was no time x forage type interaction on Cp:Pl-Cu ratio (P>0.05).

There was no forage x antagonist interaction (P>0.05) on Cp:Pl-Cu ratio an any weekly time points. There was also no effect of forage type on Cp:Pl-Cu ratio (P>0.05). However, there was an effect (P<0.05) of antagonists on Cp:Pl-Cu ratio at week 3, when lambs fed diet supplemented with Mo and S had lower Cp:Pl-Cu ratio compared with those had diet no added Mo and S, with the mean values of 0.75 and 0.77 (s.e.d, 0.101) respectively.

Table 4.18. Ceruloplasmin to plasma copper ratio of growing lambs fed diets based on forages grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Week	Treatment[2]				Significance[3]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	0.81	0.60	0.70	0.69	0.084	0.861	0.083	0.123
1	0.62	0.64	0.69	0.68	0.064	0.235	0.913	0.701
2	0.56	0.58	0.54	0.59	0.079	0.880	0.474	0.846
3	0.76	0.59	0.70	0.62	0.059	0.764	0.007	0.297
4	0.69	0.65	0.70	0.57	0.067	0.491	0.065	0.346
5	0.77	0.66	0.76	0.85	0.068	0.071	0.978	0.050
6	0.75	0.65	0.67	0.59	0.092	0.288	0.177	0.845
7	0.63	0.59	0.63	0.60	0.059	0.856	0.471	0.860
8	0.79	0.74	0.80	0.71	0.060	0.716	0.108	0.607
9	0.89	0.81	0.85	0.89	0.103	0.787	0.782	0.412
10	0.70	0.83	0.71	0.73	0.075	0.434	0.179	0.352

[1] week zero was used as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.035	<.001
Time x Forage effect	0.023	0.607
Time x Antagonist effect	0.023	0.032
Time x Forage x Antagonist effect	0.053	0.150

4.3.10. Superoxide dismutase activity

There was an effect of time on whole blood SOD activity (P<0.001) (Table 4.19). However, there was no effect (P>0.05) of time x treatment interaction on SOD activity.

There was also no effect (P>0.05) of dietary treatment on SOD activity of the lambs throughout the study.

Table 4.19. Superoxide dismutase activity in growing fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S (U/g of Hb)[1].

Week	Treatment					Significance[2]		
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	2320	2475	2320	2243	255.3	0.527	0.830	0.526
4	1691	1859	1864	1719	166.0	0.889	0.922	0.193
8	1602	1586	1618	1469	201.0	0.724	0.564	0.642
10	1836	1639	1730	1588	217.9	0.616	0.281	0.858

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	62.4	<.001
Time x Forage effect	130.0	0.716
Time x Antagonist effect	130.0	0.663
Time x Forage x Antagonist effect	150.8	0.486

4.3.11. Haematology parameters

4.3.11.1. Haematocrit

There was a trend for the effect (P<0.1) of time on Hct level (Table 4.20). There was also an effect of time x forage type interaction on Haematocrit (%) (P<0.05). However, there was no time x forage type x antagonists, or time x antagonist interaction on Hct (%) (P>0.05).

There was no forage x antagonist interaction on Hct level during the period of the study (P>0.05). At week 10, lambs supplemented with Mo and S had a lower (P<0.05) Hct (%) compared with lambs receiving no supplemented Mo and S, with the mean value of 37.63 and 39.30 % respectively.

Table 4.20. Haematocrit (%) in growing fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S[1].

Week	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
4	35.94	37.69	38.56	37.14	1.603	0.369	0.885	0.173
8	38.7	39.9	35.87	35.76	1.276	<.001	0.551	0.474
10	37.24	40.13	38.02	38.46	0.935	0.505	0.018	0.076

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.437	0.053
Time x Forage effect	0.775	<.001
Time x Antagonist effect	0.775	0.132
Time x Forage x Antagonist effect	0.925	0.525

4.3.11.2. Haemoglobin concentration

There was an effect (P<0.001) of time on haemoglobin concentration, with concentration rising by the end of the study (Table 4.21). There was also a time x forage type interaction on Hb concentration (P<0.001). However, there was no time x antagonist interaction, or time x forage type x antagonist interaction on Hb concentration (P>0.05).

There was no forage x antagonist interaction on blood Hb concentration at any weekly time points (P>0.05). There was also no effect (P>0.05) of antagonists on blood Hb concentration. At week 8, the grass haylage fed lambs had a higher (P<0.05) blood Hb concentration compared with the maize silage fed lambs. At week 10, there was also a trend (P<0.1) for higher Hb concentration in lambs fed grass haylage compared with the lambs fed maize silage.

Table 4.21. Haemoglobin concentration in growing fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S (g/dL)[1].

Week	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
0	11.12	11.88	11.80	11.61	0.446	0.517	0.377	0.146
4	13.14	13.65	13.22	13.65	0.457	0.902	0.157	0.902
8	13.63	14.08	13.05	12.88	0.359	0.002	0.586	0.233
10	14.08	15	14.11	14.10	0.357	0.097	0.084	0.078

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.135	<.001
Time x Forage effect	0.233	<.001
Time x Antagonist effect	0.233	0.438
Time x Forage x Antagonist effect	0.286	0.344

4.3.11.3. Red blood cell counts

There was no effect (*P*>0.05) of time on RBC counts (Table 4.22). However, there was an effect of time x forage type interaction on the RBC counts (*P*<0.001). There was no effect of time x forage type x antagonist interaction on RBC counts (*P*>0.05).

There was no forage x antagonist interaction on RBC counts throughout the study (*P*>0.05). However, at week 8, the GH fed lambs had a higher (*P*<0.001) RBC counts compared with the MS fed lambs (13.8 and 12.5 $10^6/mm^3$; s.e.d., 0.28 respectively). At week 10, lambs fed diets supplemented with Mo and S had a higher (*P*<0.05) RBC counts compared with those not receiving the antagonists (13.7 and 13.1 $10^6/mm^3$; s.e.d, 0.24) respectively.

Table 4.22. Red blood cell counts in growing fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S ($10^6/mm^3$)[1].

Week	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
4	13.14	13.29	13.41	13.11	0.448	0.891	0.824	0.484
8	13.70	13.83	12.53	12.50	0.395	<.001	0.858	0.770
10	13.11	13.96	13.17	13.42	0.34	0.335	0.031	0.224

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.121	0.518
Time x Forage effect	0.258	<.001
Time x Antagonist effect	0.258	0.684
Time x Forage x Antagonist effect	0.294	0.633

4.3.11.4. White blood cell counts

There was an effect (P<0.001) of time on WBC counts (Table 4.23). However, there was no time x treatment interaction on WBC counts (P>0.05).

There was no effect of dietary treatment on WBC counts throughout the study (P>0.05).

Table 4.23. white blood cell counts in growing fed diets based on grass haylage (GH) or maize silage (MS) supplemented without (-) or with (+) Mo and S ($10^3/mm^3$)[1].

Week	Treatment				Significance[2]			
	GH-	GH+	MS-	MS+	s.e.d	F	A	Int.
4	9.47	8.99	9.04	9.29	0.894	0.917	0.856	0.565
8	8.22	7.32	7.56	8.38	0.835	0.740	0.944	0.155
10	9.60	7.32	8.90	9.58	1.100	0.327	0.312	0.068

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x foragex antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.294	<.001
Time x Forage effect	0.570	0.237
Time x Antagonist effect	0.570	0.480
Time x Forage x Antagonist effect	0.664	0.255

4.4. Discussion

In the current study, DLWG in growing lambs on either forage were lower than the 200 g/d as predicted by AFRC (1993), particularly in lambs offered GH. The low DLWG in GH and MS fed lambs may be related to the lower DMI by lambs than predicted (1 kg DM/d) according to AFRC (1993) to support a weight gain of 200 g/d. As in Chapter 3, Texel cross lambs fed MS had a DLWG (0.195 g/d) that was close to 200 g/d when lambs consumed 0.95 kg DM/d. In addition, the breed difference between Chapters 3 and 4 in terms of DLWG may be contribut to the difference in DLWG, as breed effect is not accounted for in prediction equation by AFRC (1993). In the currents study, lambs offered the MS diet were heavier compared to those offered GH. However, DMI in the GH fed lambs was greater compared to MS. Similar results were observed in Chapter 3, where the MS fed lambs were heavier compared with the DGP fed lambs. Shavers *et al*. (1985) also showed that cows offered MS had a higher daily gain compared with those offered alfalfa haylage. Similarly, Coblentz *et al*. (2015) showed a significant (16%) reduction in average daily gain of cows after inclusion of alfalfa haylage at a rate of 33.3% into the diet contained of maize silage 55.8% and Alfalfa haylage 44.2%. Coblentz *et al*. (2015) also reported an increase in NDF concentration by the inclusion of haylage. The lower weight gain by GH fed lambs may be associated with its higher NDF content than MS (656 vs. 381 mg/kg DM), which results in poor forage quality of GH and reduce organic matter digestibility and dietary energy intake (Salinas-Chavira *et al*., 2013).

Similar to the results in Chapter 3, in the current study, dietary Mo and S had no effect on lamb performance and also produced no clinical symptoms of Cu deficiency, confirming findings of other authors who reported that dietary antagonists had no significant effect of lambs performance (Williams, 2004; Suttle, 2012; Sefdeen *et al*., 2016) or produce any clinical symptoms (Wentink *et al.*, 1999; Knowles *et al.*, 2000; Sefdeen *et al*., 2016).

Rumen pH was higher in lambs offered GH compared to MS. This effect may be due to the higher buffering capacity in the GH, which was found to be 1.6 time as high as that in maize silage (Shaver *et al*., 1985). The high NDF content in the GH compared with MS possibily also attributed to an elevated rumen pH in growing lambs though producing less VFA, or provision of salivary buffers as a result of increased chewing time (Yang and Beauchemin, 2007; Jalili *et al*., 2012). Moreover, GH feed particles were longer than MS, and diets with long particle size has been shown to result in animals spending more time chewing and ruminating, and increases saliva production, which in turn elevates rumen pH (Ørskov, 1987). Kmicikewycz *et al*. (2015) reported that cows that consumed a long particle size of

maize silage had a higher rumen pH compared to those fed short particle size maize silage. Therefore, the combination of effects of forage particle length and the NDF content in the diet may have caused a significant impact on ruminal pH through the excretion of salivary buffers and increased rumen pH (Jalili *et al*., 2012; Kmicikewycz *et al*., 2015).

Final liver Cu concentration of lambs whether receiving or not receiving additional Mo and S were 131 and 223 mg/kg DM respectively. These are well in excess of the 20 mg/kg DM considered to be marginal for Cu deficiency (Suttle, 2010). Compared to the whole liver Cu content in the initial slaughter group, all lambs had decreased levels which had a net negative retention, except lambs fed the maize silage diet unsupplemented with antagonists showing a positive retention and gained 0.13 mg/kg DM per day. This implies that approximately 11 mg Cu/kg DM was not sufficient to maintain liver Cu status over a period of 10 weeks study in Swaledale growing lambs on GH supplemented without or with antagonists, or MS supplemented with antagonists. Liver Cu status in the present study was higher in lambs fed MS than GH. Suttle (1980a) showed that the availability of Cu for absorption in the dried forages was higher compared with silages, although the reasons for this effect were unknown (Suttle, 1983a). Sinclair *et al*. (2017) reported that cows offered MS diet had higher liver Cu concentration compared with those offered GS diet (511 and 424 mg/kg DM) respectively, and they also reported no reasons for this difference. Moreover, in a study that investigated the effect of floor type on Cu metabolism in lambs, Crosby *et al*. (2004) reported that liver Cu concentration was higher in lambs housed on expanded floor compared with those housed on straw-bedded floor (226 and 191 mg/kg DM; s.e.d, 12.9) respectively. They hypothesised that the lower liver Cu concentration in straw-bedded lambs was possibly associated with the lower rumen acidity caused by eating straw elevating rumination and salivation. The consequence of this was reduction in overall digestibility, which in turn, promoted sulfide-reducing ciliate protozoa that reduced sulfur to sulfide and hence decreased Cu availability. Therefore, the difference in liver Cu status between forages in the current study was possibly associated with low feed quality and feed digestibility in GH and higher rumen pH by GH. Similarly, in Chapter 3, the higher liver Cu status was coupled with lower rumen pH in the lambs fed dried grass pellets compared with lambs fed MS diet.

Dietary antagonists in the present study resulted in a dramatic reduction in liver Cu concentration by 41.3% similar to the predicted (50%) reduction in Cu availability by the inclusion of 5 mg Mo/kg DM and 4 g s/kg DM using the equation of Suttle and Maclachlun (1976). The reduction of liver Cu status in the present study by the addition of Mo and S is

in accordance with the results of Chapter 3 and results that reported by Williams (2004), Cosby *et al*. (2004), Al-Kirshi *et al*. (2011) and Acharya *et al*. (2016) in sheep, and Sinclair *et al*. (2017) in cows. This is effect is due to the antagonist effect of Mo as molybdate which interacts with sulfide in the rumen producing thiomolybdates that have a high affinity to complexes which are Cu, forming insoluble Cu-TM complex and excreted via faeces (Dick *et al*., 1976). As a consequence, liver Cu concentration is depleted in order to meet tissue demand (Robinson *et al*., 1987; Suttle, 2010). Alternative hypothesis for liver Cu depletion due to the inclusion of Mo and S may be related to the systemic effect of absorbed thiomolybdates, which possibly reduce liver Cu concentration either directly by sequestering Cu from hepatocytes increasing Cu excretion (Gooneratne, 2012), or indirectly due to thiomolybdate binding with Cu with such strong affinity with Cu in the Cp protein which is not recycled back to liver and broken down, and as such half-life (2-3 d; Linder, 1991) of Cp may be altered, resulting in a reduction in liver Cu concentration (Williams, 2004). The results from the current study clearly indicated that inclusion of Cu antagonists significantly reduced Cp activity and Pl-Cu concentration compared to unsupplemented lambs. The reason for the observed reduction in the blood Cu was not clear whether it is arisen from direct effect of rumen thiomolybdates or systemic effects. Nevertheless, the reduction in Pl-Cu concentration and Cp activity due to Cu antagonists have been attributed to the direct effects of rumen thiomolybdates that cause a reduction in utilisation of dietary Cu (Suttle and Field, 1968; Zhou *et al*., 2016).

Robinson *et al*. (1987) observed that additional Mo at approximately 5 mg/kg DM into lambs diet that contained approximately 4 g/kg DM of S significantly decreased liver Cu concentration, Cp activity, and Pl-Cu concentration. The adverse effects of antagonists on liver Cu status has been attributed to the physiological rumen CuxMoxS interactions, resulting in decrease Cu availability. In the same study when the addition of dietary Mo was increased to 11 mg/kg DM a similar effect of antagonists was observed, but Pl-Cu concentration was increased. Robinson *et al*. (1987) suggested that the reduction in liver Cu status and Cp activity that accompanied increase Pl-Cu concentration may be due to the combined gut and systemic effects of absorbed thiomolybdates. Moreover, Williams (2004) attributed the reduction in Cp activity and increased Pl-Cu concentration, which was mirrored by reduction in liver Cu concentration, to systemic effect of thiomolybdate in sheep fed a diet that had a Cu:Mo ratio 1:1 or 1:2. Similarly, Mackenzie *et al*. (2008) demonstrated that offering a diet, with Cu:Mo ratio 1:1, to growing lambs resulted in a significant reduction in Cp activity and increased Pl-Cu concentration, but the effect of additional Mo was not at the Cp gene expression level. Suttle (2010) and Sinclair *et al*. (2017) suggested that dietary Cu:Mo ratio (1:1) is required for TM to be absorbed from the rumen into the blood stream and cause a systemic effect of impairment of Cu-containing enzymes. In the current study,

the dietary Cu:Mo ratio was reduced by supplementation molybdenum in both diets from approximately 7.3:1 to 2.2 :1, which was greater than 1.0.

The use of Cp:Pl-Cu ratio rather than Pl-Cu concentration or Cp activity has been proposed to be more beneficial for the detection absorbed thiomolybdate in ruminants due to high dietary Mo and S (Mackenzie *et al*., 2001). In the present study, Pl-Mo concentration was significantly increased following the addition of antagonists, but Cp:Pl-Cu ratio was not affected by dietary treatment. However, Cp:Pl-Cu ratio in both added or no added antagonists groups were lower than threshold 1.5 regarded as indicative of the presence of TM in the blood (Kendall *et al*., 2000; Telfer *et al*., 2004). This result is in consistent with the results of Sinclair *et al*. (2017) who reported that addition of Mo and S resulted in a significant increase in Pl-Mo concentration without having an impact on Cp:Pl-Cu ratio. In contrast, Williams (2004) and Mackenzie *et al*. (2008) reported that Cp:Pl-Cu ratio was significantly reduced by the inclusion of Mo and S and the reason for this effect has been linked with the systemic effect of absorbed thiomolybdates. Therefore, the antagonist effects of Mo and S on liver and blood parameters in the present study may be related to the direct effect of the rumen thiomolybdate on Cu absorption, but the systemic effect of absorbed thiomolybdate was not clear.

In the present study, the Cu status blood parameters such as Pl-Cu concentration, Cp activity, and Cp:Pl-Cu ratio were analysed to determine the antagonist effect on Cu status. The mean Pl-Cu concentration in the present study were 14.1 µmol/L, which is well above 8 - 9.4 µmol/L, regarded to be marginal for Cu deficiency (Kendall *et al*., 2000; Telfer *et al*., 2004; Suttle, 2010), and the mean value of 3-4.5 µmol/L that has been associated with clinical Cu deficiency in sheep (Whitelaw *et al*., 1983; Woolliams *et al*., 1986b). Plasma Cu has been suggested to be maintained within a normal range during depletion or repletion by changes in liver Cu concentration (Laven and Livesey, 2005). In the current study, there was no difference in Pl-Cu concentration, or Cp activity between forages. These results are in accordance with the Sinclair *et al*. (2017) who reported that Pl-Cu concentration and Cp activity were not affected in dairy cows fed grass silage or maize silage diets.

Molybdenum in the diet is absorbed as water-soluble molybdates (Suttle, 2010), which are normally stored in tissues such as liver, kidney, and adrenal gland. Molybdoprotein binds to sulphite oxidase in the mitochondrial membrane, and to dehydrogenase and aldehyde oxidase in the sytosol (Johnson, 1997). In the present study, dietary treatment had little

effect on liver Mo status. This results are consistent with the results of Acharya *et al*. (2016) who reported that administration of Mo 27.3 mg/d had no effect on lambs liver Mo concentration. Sinclair *et al*. (2017) observed a small effect of additional S and Mo on liver Mo concentration and suggested that the liver may not be a major depot of Mo in dairy cows and was unavailable for uptake by the liver. Therefore, the results of the present study also confirm that the liver is not a major depot of Mo in lambs.

4.5. Conclusion

The results of the current study demonstrated that additional Mo and S had no effect on lambs performance and feed intake. Lambs offered MS based diets were heavier compared with those offered GH. A dietary Cu concentration 11 mg/kg DM fed with grass haylage or maize silage fed to Swaledale growing lambs that had a diet containing approximately 2 mg Mo/kg DM and S 2 g/kg DM without or with supplemented Mo and S was not enough to maintain liver Cu status over a period of 10 weeks as evidenced by a net reduction in liver Cu retention in all lambs on all dietary treatment except lambs fed maize silage unsupplemented with antagonists. Liver Cu status was higher in lambs fed MS compared with the lambs fed GH. This was in accordance with a lower rumen pH in the MS fed lambs than the GH fed lambs. Liver Cu and blood Cu (including plasma Cu concentration, and ceruloplasmin actively) status in growing lambs were significantly reduced by inclusion Mo and S, and this effect was probabely due to the direct effect of rumen TM on reducing Cu availability rather than systemic effect of absorbed thiomolybdate. To conclude, there was no interaction between forage type and antagonists on liver Cu status. The higher liver Cu status in the MS fed lambs was possibily related to a low rumen pH in these lambs, although, more research is needed to elucidate the role of the rumen on Cu antagonism and the effects of rumen pH on Cu metabolism.

Chapter 5 The effects of forage type on copper distribution between rumen digesta fractions and the involvement of the rumen digesta fractions in the interactions between copper, molybdenum, and sulfur

5.1. Introduction

The availability of copper (Cu) for absorption in ruminants is not necessarily dependent on the mineral concentration in the diet, as the solubility of Cu in the digestive tract is also a prerequisite for absorption (Bremner, 1970). Copper in the rumen digesta has been found to be mostly (above 87%) associated with the solid phase, which includes undigested plant material, protozoa, and bacteria (Allen and Gawthorne, 1987; Waghorn, 1990). This association reduces Cu solubility and hence decreases Cu available for absorption (Price and Chester, 1985; Price *et al*., 1987). Therefore, this would suggest the importance of the involvement of the solid phase of rumen digesta and the distribution of Cu between rumen digesta fractions in Cu utilisation by ruminants.

In Chapter 3, liver Cu status was higher when dried grass pellet diet was offered compared with maize silage, while in Chapter 4, maize silage showed a higher liver Cu status compared with grass haylage. Dietary Cu intake in both Chapters was similar at 8.2 and 8.8 mg of Cu/d respectively. The availability of Cu from dried forages has been found to be greater than fresh herbage and silages (Fisher *et al*., 1972; Suttle, 1980a; 1983b). In addition, the antagonist effect of Mo and S on Cu metabolism had been reported to be less in preserved forages as hay or silage (Suttle, 1986; Suttle, 2010). The reason for the differences in Cu metabolism between different forages was not clear, but the higher Cu absorption from dried forges has been associated with the lower release of Cu by these diets into the rumen, the site of Cu-Mo-S interaction (Suttle, 1983b). Waghorn *et al*. (1990) also showed that dried forages fed to sheep had a lower proportion of Cu present in the rumen supernatant fraction compared with fresh forages. Therefore, the distribution of Cu between rumen digesta fractions may be important as a possible explanation for the difference in Cu availability between forages.

The effects of Cu antagonists on Cu status of growing lambs in Chapter 3 and 4 were clearly identified, although, the reason for this effect was not clear either due to the direct effect of rumen thiomolybdates on reducing the availability of Cu for absorption (Suttle, 2010), or due to a systemic effect of absorbed thiomolybdates sequestering Cu from liver and increaseing Cu excretion (Mason, 1986). The production of thiomolybdate in the rumen and its adverse effect on Cu utilisation via producing insoluble Cu-thiomolybdate complex has

been suggested as a hypothesis (Dick *et al*., 1975; Suttle, 1991). Grace and Suttle (1979) and Allen and Gawthorne (1987) reported the interaction between Cu and Mo in the rumen was associated with the solid phase, as they showed that that most of the Mo and Cu in the rumen digesta were associated with the solid phase. Price *et al*. (1987) also demonstrated that thiomolybdates, mostly tri or tetra, were found to be mainly associated with the solid phase, and they are more likely associated with inhibiting Cu utilisation (Price *et al*., 1987). The association of Cu and thiomolybdate with the solid phase of the rumen digesta, therefore, suggests that intraruminaly Cu-thiomolybdate complexes may form (Gould and Kendall, 2011). It has been reported that Cu association with the solid phase increased by the addition of Mo and S at the expense of reduced Cu distribution in the supernatant fraction (liquid phase) (Allen and Gawthorne, 1987), which is evidenced of Cu-thiomolybdate complex in the rumen by which Cu availability diminished.

As shown in Chapters 3 and 4, forage type had a different effect of Cu metabolism and the addition of Mo and S substantially reduced liver Cu status in growing lambs. Therefore, the aims of this study were to investigate the effect of forage type on Cu distribution in rumen digesta, and the interaction between Cu, Mo, and S.

5.2. Materials and methods

5.2.1. Experimental design and basal diets

The study was conducted using a batch culture *in vitro* gas production technique (Theodorou *et al*., 1994; Sinclair *et al*., 2005), which is widely accepted for evaluating fermentation kinetics characteristics. This technique is similar to other *in vitro* technique using substrate, anaerobic media and a rumen fluid as inoculum, except, the incubations are in gas-tight culture vessels, where produced gas accumulates in the head-space as fermentation proceeds. The experiment was designed as a 4 x 2 factorial. Forages used in the culture were maize silage (MS), dried grass pellets (DGP), and grass haylage (GH) that were used in Chapter 3 and 4, plus grass silage (GS). The chemical composition of all forages (DM, ash, CP, NDF, and EE) was analysed as described in Chapter 2 (section 2.1.1 to 2.1.5). Dietary mineral content of the experimental forages was also determined as described in section 2.4.1. The chemical composition and mineral content of forages used in current study as shown in Table 5.1.

Table 5.1. Chemical and mineral composition of the grass silage (GS), maize silage (MS), dried grass pellets (DGP), and grass haylage (GH) used in the experiment.

Items	Forage			
	GS	MS	DGP	GH
Chemical composition, g/kg DM				
DM, g/kg	nd[1]	324	889	868
CP,	134	73	184	96
NDF,	421	381	426	654
EE,	32	34	29	11
Ash,	86	37	84	63
NSC[2],	327	475	277	176
Mineral composition, mg/kg DM				
Cu,	7.6	4.7	9.6	6.3
Mo,	1.27	0.52	0.94	1.40
S, g/kg DM	2.3	0.92	3.6	1.4

[1] nd = not determined.

[2] Non-structural carbohydrate (NSC) was calculated by subtracting the sum of the amounts (g/kg) of CP, NDF, EE, and ash from 1000 (McDonald *et al*., 2011)

All forages were freeze dried and milled to pass through a 3 mm mesh. The four forages were then either un-supplemented, or supplemented with 5 mg Mo/kgDM and 2 g S/kg DM in order to evaluate Cu distribution within fermented rumen fluid fractions. The additional antagonists (Mo and S) were dissolved in purite water and then 1 ml of this was added into treatment vessels. The nitrogen content of the mineral mix was balanced with feed grade urea, after the urea had been dissolved in purite water and then 1 ml of this solution was added to control bottles. The added Mo was in the form of ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Fisher Scientific, Leicester, UK), and S was in the form of ammonium sulfate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA). The added urea was (Trouw Nutrition, Cheshire, UK). Dietary treatments are present in Table 5.2.

Table 5.2. Dietary treatments.

Treatment	Forage type
DGP-	Dried grass pellets, no antagonists
DGP+	Dried grass pellets, supplemental Mo + S
GH-	Grass Haylage, no antagonists
GH+	Grass Haylage, supplemental Mo + S
MS-	Maize silage, no antagonists
MS+	Maize silage, supplemental Mo + S
GS-	Grass silage, no antagonists
GS+	Grass silage, supplemental Mo + S

5.2.2. Inoculum

One day prior to the start of the experiment, an artificial saliva (9.8 g/l $NaHCO_3$, 9.3 g/l $Na_2HPO_4.12H_2O$, 0.47 g/l NaCl, 0.57 g/l KCl, 0.04 g/l $CaCl_2$ anhyd., 0.06 g/l $MgCl_2$ anhyd) was made using purite water (McDougall, 1984). All chemicals used in constituting the buffers solution were purchased from Sigma Aldrich, UK. The saliva was then autoclaved at 120˚C for 30 min and stored at 39°C. Approximately 2 grams (DM) of each experimental forage were weighed into 250 ml pyrex bottles. Bovine animal rumen fluid (RF) (approximately 5 l in total) was obtained from a freshly slaughtered cow at the ABP abattoir (Shrewsbury, UK), and placed in a pre-warmed thermos, filled to the top so as to minimise the risk of aerobic infusion and sealed before being transported to the lab for processing. Within approximately 45 min of collection, the rumen fluid was filtered through four layers of muslin cloth into an 8 L conical flask containing of artificial saliva, to create a desired ratio of 60:40 (v/v) (RF : saliva), and placed in the water bath at 39°C and purged with CO_2. The initial pH of the RF: saliva mixture was then determined as described in Section 2.1.6. For all treatments 200 ml of RF: Saliva (60:40, v/v; pH 7.02) mixture was added to each bottle. The bottles were then sealed with adapted washer caps (R.H Nuttall LTD, Birmingham, UK) (Fig. 5.1) and incubated at 39°C. Four blank bottles were also incubated in each run in order to correct gas production. All treatments were incubated in quadruplicate (at 12th of May 2015) and replicated over four separate periods. The experimental design had 8 treatments with 4 replicates per treatment (32 in total) in each of four periods.

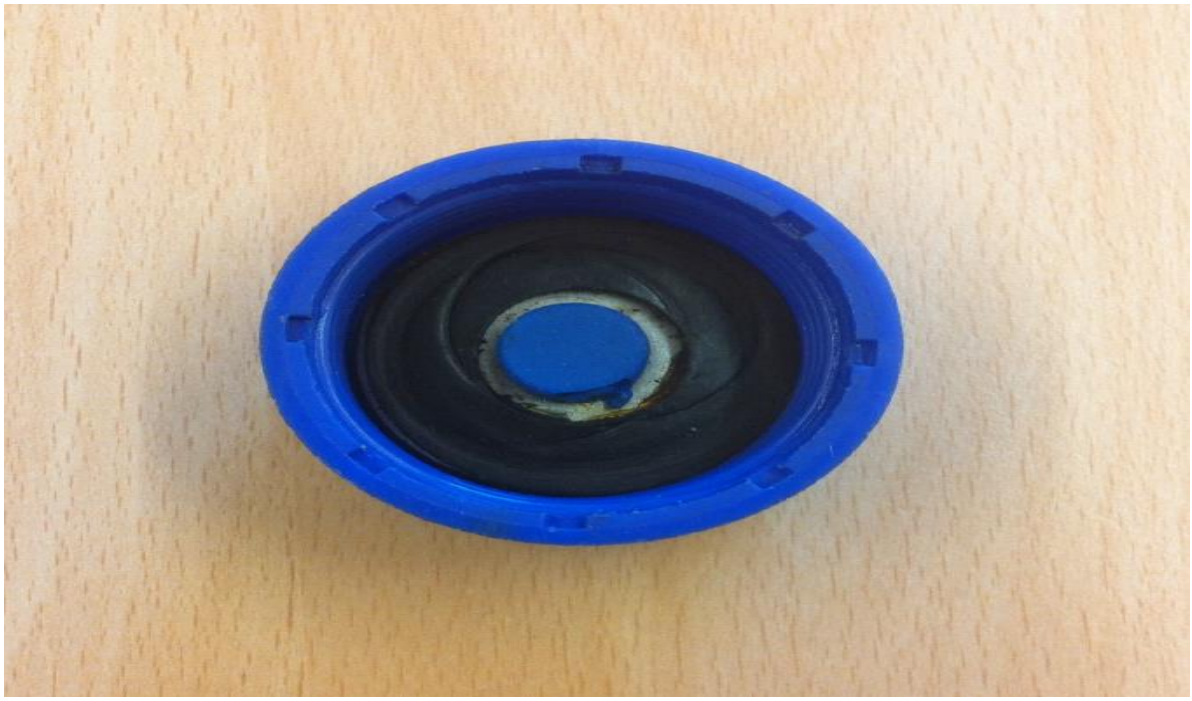


Figure 5.1. Adapted washer cap

The accumulated head-space gas pressure was measured manually at 0, 3, 6, 12, 18, 24, 30, 36, 42, and 48 hrs, after the addition of substrates using a pressure transducer (T443; Bailey and MacKay Ltd., Birmingham UK), and the gas released at each time point until the head-space returned to ambient pressure. The gas production pressure (kPa) data was corrected for the substrate blank and transferred to ml according to Purcell *et al*. (2011) as:

$$\text{Gas production (ml)} = \frac{\text{Vh}}{\text{pa}} x \text{ Pt}$$

Where Vh is bottle headspace volume (107.55 ml), Pa is atmospheric pressure (101.4 kPa) and Pt the gas pressure of a transducer (kPa). The cumulative gas production (ml/g DM) was determined per substrate fermented as cumulative volume per gram of DM incubated (Calabro *et al*., 2005).

5.2.3. Vessel pH determination

At the end of each run (after 48 hrs) the lids were removed from all vessels and pH was directly determined as described in a section (2.1.6).

5.2.4. Fractionation of vessel fluid

Following pH determination, the vessels were chilled in a freezer for approximately 1 hr to inhibit microbial activity. Vessel contents were then separated into four different fractions using the methods adapted from Price and Chester (1985) and Allen and Gawthorne, (1987). The first fraction, strained-solids fraction (SS), containing plant material, adherent bacteria, and protozoa, retained by straining the vessel content through sintered crucible with porosity 1 (Fisher Scientific Ltd, Leicestershire, UK). The second fraction, protozoa-rich fraction, consisting of the precipitate obtained by centrifugation (1000 g for 10 min; Sigma 3-16 KL, Germany) of the fluid that passed through sintered crucible in the first fraction. The third fraction, bacterial-rich fraction, consisting of the precipitate obtained by centrifugation (25000 g for 30 min; Rotina 46 R Hettich Zentrifugen, Germany) of the supernatant fraction from the second fraction. This fraction consisted of bacteria and very fine plant particles. The fourth fraction, supernatant fraction (SN), consisted of the supernatant obtained from the third fraction, and contained the soluble component of the vessels content.

5.2.5. Mineral analysis of vessels fractions

Samples from all collected fractions, except SN, were oven dried at 60°C for 48hrs. Approximately 0.5 g of dried SS fraction and all dried samples of PR and BR fractions were digested and analysed for mineral content as described in section 2.4.1. Samples of the SN fraction were directly analysed for mineral content as described in section 2.4.1. Rumen fluid:saliva mixture was not analysed for mineral content.

5.2.6. Statistical analysis

Repeated measures analysis of variance was used to analyses cumulative gas production as a 4 x 2 factorial design with the main effects being forage type (F), antagonists (Ant.), and forage x antagonist interaction (Int.). Vessel pH and mineral distribution were also analysed as a 4 x 2 factorial design with main effects of forage type (F), antagonists (Ant.), and forage x antagonists' interaction (Int.). Runs were used as a blocking factor. Analysis was conducted using Genstat 17th edition (Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Significance was set at $P<0.05$ and trends at $P<0.10$. Significance differences between means were tested using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

5.3. Results

5.3.1. Gas production

Repeated measures analysis indicated that there was an effect of time (P<0.001) on cumulative gas production, with increasing gas production amongst all forages over the period of the study (Table 5.3). Similarly, there was a time x forage interaction on cumulative gas production (P<0.001). Maize silage had the highest cumulative gas production over the period of the study, followed by grass silage and dried grass pellets, while the lowest gas production was in the grass haylage forage, with mean values being 16.4, 15.0, 13.5, and 11 ml/g DM (s.e.d, 0.830) respectively. There was no time x forage x antagonist interaction, or time x antagonist interaction on cumulative gas production (P>0.05).

There was a forage x antagonist interaction (P<0.001) on cumulative gas production throughout the study period (Table 5.3). Compared to unsupplemented antagonists, the addition of antagonists reduced (P<0.05) cumulative gas production in maize silage throughout the study, whereas in other forages the addition of antagonists had no effect (P>0.05) on cumulative gas production.

There was an effect of forage type on cumulative gas production (P<0.001). At time 3hrs to 12hrs, cumulative gas production was higher in maize silage, followed by grass silage, then in dried grass pellets, and lowest in grass haylage. At 18hrs and 24hrs, cumulative gas production was higher in maize silage and grass silage, intermediated in dried grass pellets, and lowest in grass haylage. At 30hrs until 48hrs, cumulative grass production was higher in maize silage, intermediate in grass silage and dried grass pellets, and lowest in grass haylage. There was no effect of Mo and S on cumulative grass production throughout the study (P>0.05).

Table 5.3. The cumulative gas production of the grass silage (GS), maize silage (MS), dried grass pellets (DGP), and grass haylage (GH) supplemented without or with molybdenum and sulfur[1].

Time, hour	Treatment[2]									Significance[3]		
	GS-	GS+	MS-	MS+	DGP-	DGP+	GH-	GH+	s.e.d	F	A	Int.
3	3.6^{d}	3.3^{cd}	4.4^{e}	3.4^{d}	2.8^{bc}	3.1^{cd}	2.2^{a}	2.4^{ab}	0.27	<.001	0.173	0.007
6	6.4^{d}	6.2^{d}	8.9^{e}	6.8^{d}	4.8^{bc}	5.7^{cd}	3.1^{a}	3.9^{ab}	0.55	<.001	0.555	<.001
12	11.1^{d}	11.1^{d}	13.7^{e}	10.7^{cd}	8.6^{b}	9.5^{bc}	5.8^{a}	7.0^{a}	0.67	<.001	0.506	<.001
18	14.7^{d}	14.8^{d}	17.5^{e}	13.2^{cd}	11.4^{bc}	12.9^{cd}	8.3^{a}	10.3^{ab}	1.01	<.001	0.753	<.001
24	17.6^{d}	16.2^{cd}	20.6^{e}	16.1^{cd}	14.0^{bc}	16.0^{cd}	11.3^{a}	13.2^{ab}	1.27	<.001	0.436	<.001
30	19.9^{d}	18.2^{cd}	23.0^{e}	19.4^{d}	16.4^{bc}	18.3^{cd}	13.4^{a}	15.4^{ab}	1.20	<.001	0.566	0.002
36	21.5^{d}	19.6^{cd}	24.8^{e}	21.0^{d}	18.2^{bc}	20.1^{cd}	15.1^{a}	16.9^{ab}	1.26	<.001	0.440	0.003
42	22.6^{d}	20.6^{bcd}	26.1^{e}	22.3^{d}	19.6^{bc}	21.4^{cd}	16.5^{a}	18.2^{ab}	1.30	<.001	0.386	0.004
48	23.5^{d}	21.2^{bcd}	27.2^{e}	23.2^{cd}	20.5^{bc}	22.4^{cd}	17.7^{a}	19.3^{ab}	1.36	<.001	0.311	0.005

[1] F= main effect of forage type; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[2] ^{a,d,c,d,e} Means within a row with different superscripts are significantly different (P<0.05).

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.16	<.001
Time x Forage effect	0.77	<.001
Time x Antagonist effect	0.55	0.439
Time x Forage x Antagonist effect	0.83	0.1

5.3.2. Vessels pH

There was no forage x antagonist interaction (P>0.05) on the final vessel pH (Fig. 5.2). There was also no effect (P>0.05) of antagonists on the final vessel pH. However, forage type had an effect on the final vessels pH (P<0.001), with maize silage resulting in the lowest pH (6.35), followed by grass silage (6.46) and the highest pH was in both dried grass pellets (6.49) and grass haylage (6.51) (s.e.d, 0.012).

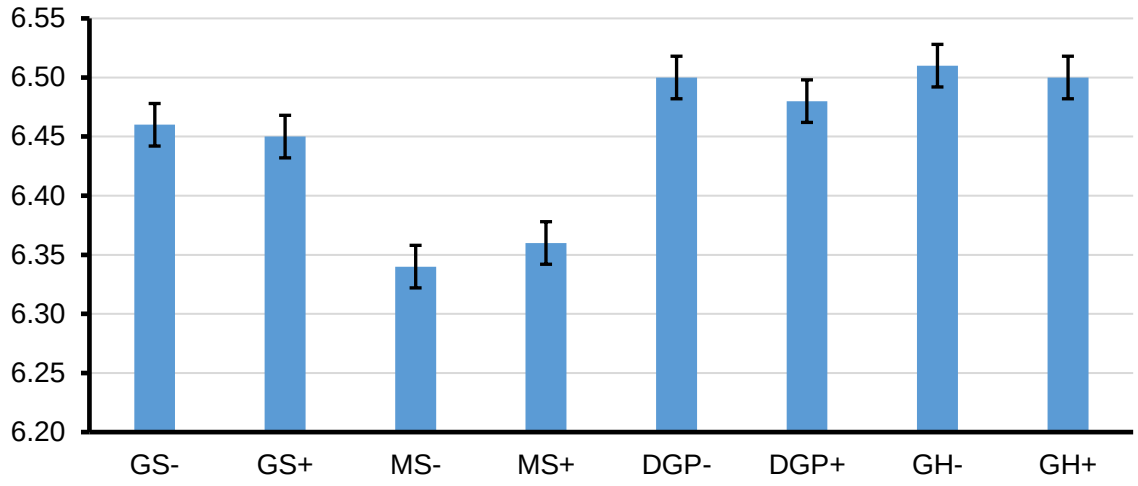


Figure 5.2. The effect of forage type grass silage (GS), maize silage (MS), dried grass pellets (DGP), and grass haylage (GH) supplemented without (-) or with (+) Mo and S on final vessels pH during the 48hrs of in vitro incubation at 39°C. Error bars indicate SED.

5.3.3. Distribution of minerals within fluid fractions

5.3.3.1. Copper distribution

In strained solid fraction, there was no forage x antagonist interaction (P>0.05) on the percentage of Cu distribution (Table 5.4). There was also no effect of Cu antagonists on the percentage of Cu distribution in this fraction (P>0.05). However, Cu proportion in strained solid fraction was different (P<0.001) between forages, with dried grass pellets having a higher Cu proportion (45.0%), followed by grass silage (38.5%) then maize silage (35.0%), and grass haylage had the lowest Cu proportion (26.7%) (s.e.d, 1.35).

In the protozoa rich fraction, there was no forage x antagonist interaction (P>0.05) on the percentage of Cu distribution (Table 5.4). However, there was an effect (P<0.001) of the forage type on the of Cu distribution, the highest proportion of Cu was in grass haylage (32.4%), and the lowest proportion was in the dried grass pellets (23.9%), while both grass silage and maize silage were intermediate (27.4% and 27.1% respectively) (s.e.d, 0.86). The addition of the Mo and S (P<0.05) increased the percentage of Cu associated with the protozoa rich fraction compared with no antagonists (28.6% and 26.8%; s.e.d, 0.61) respectively.

There was a forage x antagonist interaction (P<0.05) on the proportion of Cu in the bacterial rich fraction (Table 5.4). The addition of Mo and S increased (P<0.05) the proportion of Cu grass haylage supplemented with antagonists compared with no added antagonists, whilst other forages were not affected by addition of Mo and S (P>0.05). The proportion of Cu in both grass haylage (28.4%) and maize silage (27.0%) was higher (P<0.001) compared with grass silage (24.2%) and dried grass pellets (23.0%) (s.e.d, 0.81). There was no effect of antagonists on the proportion of Cu in bacteria rich fraction (P>0.05).

In the supernatant fraction, there was a forage x antagonist interaction (P<0.001) on the proportions of Cu (Table 5.4). In grass silage, maize silage, and grass haylage forages additional Mo and S reduced (P<0.05) the proportion of Cu compared with no added antagonists, while in DGP the proportion of Cu was not affected (P>0.05) by Cu antagonists. The proportion of Cu in supernatant fraction was different (P<0.001) between forages and the highest proportion of Cu was in grass haylage (12.6%), followed by maize silage (10.9%), and then grass silage (10.0%), while the lowest proportion of Cu was in dried grass pellets (8.2%) (s.e.d, 0.35).

Table 5.4. The effect of forage type supplemented without (-) or with (+) additional Mo and S on the percentage distribution of copper (%) in different fractions of *in vitro* fermented rumen fluid[1].

Rumen fluid[3] fractions	Treatment									Significance[2]		
	GS-	GS+	MS-	MS+	DGP-	DGP+	GH-	GH+	s.e.d	F	A	Int.
SS	37.8	39.1	35.2	34.8	44.1	45.8	29.1	24.2	1.91	<.001	0.541	0.058
PR	26.9	27.9	26.6	27.7	23.6	24.2	30.0	34.7	1.22	<.001	0.003	0.068
BR	24.0^{ab}	24.3^{b}	26.0^{bc}	28.0^{c}d	24.0^{ab}	21.9^{a}	27.1^{c}	29.7^{d}	1.14	<.001	0.238	0.020
SN	11.3^{c}	8.6^{ab}	12.3^{c}	9.5^{b}	8.2^{a}	8.1^{a}	13.7^{d}	11.4^{c}	0.50	<.001	<.001	<.001

[1] Forage type= grass silage (GS), maize silage (MS), dried grass pellets (DGP), grass haylage (GH).

[2] F= main effect of forage type; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[3] SS= strained solid fraction, PR= protozoal rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[4] ^{a,b,c,d} Means within a row with different superscripts are significantly different (P<0.05).

5.3.3.2. Molybdenum distribution

In strained solid fraction, there was a forage x antagonist interaction (P<0.05) on the percentage of Mo distribution (Table 5.5). Compared with forages unsupplemented with antagonists, the inclusion of Mo and S increased (P<0.05) the percentage of Mo distribution in grass silage, maize silage, and dried grass pellets, without having an effect (P>0.05) in grass haylage. The proportion of Mo was higher in forages supplemented with antagonists compared with forages with no added antagonists, with mean values of 37.3% and 33.3% respectively. There was an effect (P<0.001) of forage type on Mo proportion, with dried grass pellets having the highest Mo proportion (43.6%), followed by grass silage (37.5%), and then maze silage (34.7%), whilst the lowest Mo proportion was in grass haylage (25.3%) (s.e.d, 1.10).

In the protozoa rich fraction, there was a forage x antagonist interaction (P<0.05) on the percentage of Mo distribution (Table 5.5). The addition of Mo and S only in grass haylage resulted in an increase (P<0.05) in Mo proportion in protozoa compared with no added antagonists, whilst the Mo proportion was not affected by addition of antagonists (P>0.05). Mo proportion in protozoa was higher (P<0.001) in forages supplemented with antagonists compared with forages no added antagonists, with mean values of 28.4% and 27% respectively. Similarly, the proportion of protozoa Mo was different between forages (P<0.001). The proportion of protozoa Mo was higher in grass haylage (33.4%), intermediate in both maize silage (27.4%) and grass silage (26.5%), and the lowest in dried grass pellets (23.3%) (s.e.d, 0.80).

In the bacterial rich fraction, there was no forage x antagonist interaction (P>0.05) on the percentage of Mo distribution (Table 5.5). However, forages supplemented with antagonists had a lower proportion of bacteria Mo compared with the forages unsupplemented with antagonists, with mean values of 27.6% and 32% (s.e.d, 0.57) respectively. Likewise, there was an effect of forage type on the percentage of Mo distribution (P<0.001). The proportion of bacteria Mo was higher in grass haylage (33.3%), followed by maize silage (31.2%), and then grass silage (28.9%), and the lowest proportion was in dried grass pellets (25.9%) (s.e.d, 0.81).

In the supernatant fraction, there was a forage x antagonist interaction (P<0.001) on the percentage of Mo distribution, where additional Cu antagonists reduced (P<0.05) Mo proportion in both dried grass pellets and maize silage, while grass silage and grass haylage were not affected by addition of antagonists (P>0.05) (Table 5.5). There was a lower (P<0.001) proportion of supernatant Mo in forages supplemented with antagonists compared with forages with no added antagonists, with mean values of 6.8% and 7.7% respectively. The proportion of supernatant Mo in grass haylage was higher (P<0.001) compared with other forages.

Table 5.5. The effect of forage type supplemented without (-) or with (+) additional Mo and S on the percentage distribution of molybdenum (%) in fractions of *in vitro* fermented rumen fluid[1].

Fluid[2] fractions	Treatment									Significance[3]		
	GS-	GS+	MS-	MS+	DGP-	DGP+	GH-	GH+	s.e.d	F	A	Int.
SS	35.8^{bc}	39.2^{d}	32.8^{b}	36.7^{cd}	39.4^{d}	47.8^{e}	25.3^{a}	25.4^{a}	1.55	<.001	<.001	0.004
PR	25.6^{bc}	27.5^{cd}	27.0^{cd}	27.8^{d}	23.9^{a}	22.7^{ab}	31.3^{e}	35.4^{f}	1.13	<.001	0.015	0.013
BR	31.4	26.3	32.3	30.0	28.9	22.8	35.5	31.0	1.15	<.001	<.001	0.115
SN	7.2^{bcd}	7.0^{bc}	7.9^{de}	5.5^{a}	7.8^{cde}	6.6^{b}	8.0^{de}	8.2^{e}	0.42	<.001	<.001	<.001

[1] Forage type= grass silage (GS), maize silage (MS), dried grass pellets (DGP), grass haylage (GH).

[2] SS= strained solid fraction, PR= protozoal rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[3] F= main effect of forage type; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

[4] ^{a,b,c,d,e,f} Means within a row with different superscripts are significantly different (P<0.05).

5.3.3.3. Sulfur distribution

There was no forage x antagonist interaction (*P*>0.05) on the percentage of S distribution in any fractions of the fermented rumen fluid (Table 5.6).

In the strained solid fraction, sulfur distribution was higher in dried grass pellets (31.3%), intermediate in both maize silage (26.1%) and grass silages (24.5%) and the lowest in grass haylage (22.6%) (s.e.d, 0.81). The proportion of strained solid S in the unsupplemented forages was higher (*P*<0.001) compared with supplemented forages, with mean values of 27.4% and 24.95 (s.e.d, 0.58) respectively.

The percentage of S distribution in the protozoa rich fraction was higher (*P*<0.001) in grass haylage compared with other frages. The addition of antagonists increased (*P*<0.05) protozoa S proportion, with unsupplemented and supplemented forages having mean values of 25.2% and 28.1% (s.e.d, 1.33) respectively.

In the bacteria rich fraction, there was no effect (*P*>0.05) of addition of antagonists on S distribution in bacterial fraction (Table 5.9). The higher proportion of bacteria S was in grass silage (37.7%) and maize silages (26.2%), dried grass pellets (33.7%) being intermediate and the lowest proportion was in grass haylage (28.1%) (s.e.d, 1.94).

There was no effect (*P*>0.05) of dietary treatment on S distribution in the supernatant fraction.

Table 5.6. The effect of forage type supplemented without (-) or with (+) additional Mo and S on the distribution of sulfur (%) in different fractions of *in vitro* fermented rumen fluid[1].

Fluid[2] fractions	Treatments									Significance[3]		
	GS-	GS+	MS-	MS+	DGP-	DGP+	GH-	GH+	s.e.d	F	A	Int.
SS	25.4	23.6	26.6	25.5	32.4	30.3	25.1	20.1	1.15	<.001	<.001	0.100
PR	22.9	25.0	23.1	26.1	20.7	23.0	34.1	38.2	2.66	<.001	0.032	0.952
BR	37.6	37.7	37.3	35.0	33.6	33.7	28.1	28.1	2.74	<.001	0.689	0.915
SN	14.1	13.7	13.0	13.4	13.3	13.1	12.8	13.7	0.51	0.128	0.553	0.235

[1] Forage type= grass silage (GS), maize silage (MS), dried grass pellets (DGP), grass haylage (GH).

[2] SS= strained solid fraction, PR= protozoal rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[3] F= main effect of forage type; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

5.4. Discussion

Fermentation kinetics of soluble and insoluble fractions can be determined using the gas production technique described by Pell and Schofield (1993), Theodorou *et al*. (1994) and Cone *et al*. (1996). When feedstuffs are fermented *in vitro*, the gas produced (mainly CO_2 and CH_4) originated primarily from the fermentation of carbohydrates and protein to a lower extent (Getachew *et al*., 1998). Hence, the feedstuffs with higher carbohydrates should produce a higher amount of gases with a lower pH. In the current study, non-structural carbohydrate (NSC) content was different between forages, with MS being a higher NSC compared to other forages, with the lowest content of NSC was in GH. This may result in MS resulting in a higher gas production and a lower pH compared to GS and DGP with the lowest gas production was in GH. Brown *et al*. (2002) reported that MS (over a period of 72h) fermented quicker and produced more gas compared with GS or hay (255, 232, and 226 ml; s.e.d, 11.95) respectively. The low gas production by hay was attributed to the high level of structural carbohydrates, causing a slower fermentation (Brown *et al*., 2002). The content of NDF in MS was lower than GS and DGP by approximately twice the value of GH, which explains the difference in gas production and vessl pH between forages. As a consequence, a lower NDF content result in an increased rate of degradation of carbohydrate by microbes in the MS forage (McDonald *et al*., 2011). Moreover, García-Rodríguez *et al*. (2005) observed a higher cumulative gas production and lower pH in fermented MS compared to GS, and these differences between silages were attributed to different fermentation patterns, suggesting that maize fermented faster and to a greater extent (García-Rodríguez *et al*., 2005). In Chapter 4, the rumen pH of growing lambs fed MS was lower compared with GH. Therefore, the difference in gas production and pH may be related to differences in the NSC and NDF content between the forages.

In the current study, additional Mo and S significantly reduced gas production in the MS forage, without having an effect on the other forages. The reason for this was not clear, but may be due to the decrease in sulfide gas production following the addition of Mo, hence this reduction may contribute to an overall reduction in gas production in silage forages supplemented with antagonists. Molybdenum is reported to be poison general bacterial and micro-organism metabolism (Bryden and Bray, 1972). In addition, sulfate and molybdate are both tetra-hedral aions with the same charge which would suggest that they may be antagonistic due to the similar chemical parameters (Huisingh *et al*., 1973). Kung *et al*. (2000) demonstrated a reduction in hydrogen sulfide production by 77% after the addition of Mo at 0.25 g (fluid basis).

The release of minerals into the liquid phase (supernatant) from feedstuffs during digestion in the rumen fluid is desirable for absorption by animals, while minerals not released from digested fibrous residue of the feed may be not available for absorption (Playne *et al*., 1978). Minerals in the digestive tract in order to be absorbed must be in a soluble form (Bremner, 1970), although the solubility of minerals may be decreased owing to the formation insoluble complexes (Forth, 1973; Allen and Gawthorne, 1987). In addition, Price and Chester (1985) reported that Cu associated with solids, probably of bacterial origin, made the great contribution to the pool of available Cu in the duodenum. In the current study, the proportion of Cu in the supernatant and bacteria rich fractions was higher in MS compared with GS and DGP. Therefore, theoretically the availability of Cu from MS forage may be greater than GS or DGP. In dairy cows, Sinclair *et al*. (2017) reported that cows fed MS in the absence of antagonists had a higher liver Cu status compared with those fed GS. However, results in Chapter 3 showed that lambs fed DGP had a higher liver Cu status compared with those fed MS and contradicting that discussed above. The reason for a higher liver Cu status in the DGP fed lambs in Chapter 4, may be partially attributed to the higher out flow rate of the DGP due to its lower feed particle sieze (Thomson and Beever, 1979; Mason, 1990), resulting in less Cu exposed to the rumen antagonist interactions, increasing Cu availability (Suttle, 1991). The higher pH in DGP vs. MS in the current study supports the lower rumen pH in DGP vs MS in Chapter 3 that was suggested to be caused by the higher out flow rate of DGP

In the current study, the proportion of Mo in the supernatant fraction was higher in GH compared with MS, suggesting the higher Mo uptake from GH, confirming the higher liver and plasma Mo status in lambs fed GH compared with MS in Chapter 4. In contrast, the higher Cu distribution in the supernatant fraction of GH vs. MS contradicts the lower liver Cu status observed in lambs fed GH compared with MS. The reason for a higher mineral presence in the supernatant fraction of GH compared with other forages was not clear. However, the higher pH in GH may have encouraged the activity of rumen micro-organisms, particularly protozoa, and increased protozoa population (Ørskov, 1987; McDonald *et al*., 2011), and thus enhanced the degradation of fibre (Belanche *et al*., 2016) and breakdown of cellulose in the plant cell wall (Williams and Withers, 1991; Lee *et al*., 2000). Minerals in the cell plants are found mainly associated with the cell wall (Whitehead *et al*., 1985; Ibrahim *et al*., 1990). As a consequence, more minerals may be released from GH into the supernatant fraction compared with other forages.

Bremner (1970) demonstrated that water the solubility of Cu, in rumen content samples collected from sheep maintained on dried grass, was very low (18.8%) despite of the fact that over 80% of the dietary Cu in dried grass was water soluble, and concluded that Cu may be associated or incorporated into microbial proteins. Ward and Spears (1993) also demonstrated that rumen solubility of Cu was markedly decreased after ruminal incubation (24h) of orchard grass. Allen and Gawthorne (1987) and Price *et al*. (1987) investigated the importance of changes in Cu distribution and solubility within the ruminant alimentary tract and its influence on the utilisation of Cu, which is affected by a number of known factors without suggesting clear mechanisms by which these operate. Price and Chester (1985) showed that the relative Cu availability in rumen digesta, collected from sheep fed dried grass, and given to rats was substantially (12%) lower than that in dried grass given to sheep (75%) and they concluded that factors limiting Cu utilisation were associated mainly with the solid phase of the digesta. The results of the current study indicated that Cu, Mo, and S (above 85%) were present in the solid phase (strained solid, bacteria rich, and protozoa rich fraction) on the expense of the supernatant fraction (liquid phase). These results concur with the findings by Allen and Gawthorne (1987) and Waghorn *et al*. (1990) who demonstrated that Cu (above 87%), Mo, and S present in rumen digesta were found associated with the solid phase. Similarly, Grace and Suttle (1979) reported in the rumen digesta Mo was predominately associated with solid phase (undigested plant, bacteria, and protozoa). Price *et al*. (1987) demonstrated that in the rumen digesta of sheep maintained on dried grass, thiomolybdates (di and tri-thiomolybdate) were found predominately in the solid phase. It has been reported that thiomolybdates in the rumen digesta are temporary intermediates by associating with the solid phase to impart some stability (Gawthorne *et al*., 1985; Suttle, 1991), as thiomolybdates present in the liquid phase, if they are unbound or not absorbed, are possibly dehydrolysed (Gould and Kendall, 2011). Therefore, the presence of the majority of Cu, Mo and S in association with solid phase could facilitate the formation insoluble Cu-thiomolybdate complexes in the rumen (Gould and Kendall, 2011) and reduce Cu availability (Suttle, 2010).

In the current study the addition of Mo and S caused several changes in the proportion of Cu distribution in the fermented rumen fractions. For example, the addition of Mo and S significantly reduced Cu proportion in the SN fraction by increasing Cu association with the solid phase. Allen and Gawthorne (1987) reported that *in vitro* addition of 5 mg Mo/kg DM as tetra-thiomolybdate resulted in a substantial reduction in Cu proportion in the SN fraction, where the proportion of Cu reduced from 11.8% to 4.8% (s.e.d; 0.8) compared with no added antagonists. Price and Chester (1985) reported that samples of rumen content, collected from sheep fed dried grass diet supplemented with Mo (11.6 mg/kg DM), given to Cu-deficient rats resulted in a substantial decrease in available Cu for restoration of the

activity of cytochrome *c* oxidase in the intestine of rats. In Chapter 3 and 4, the addition of Mo and S markedly reduced liver Cu status of lambs fed forages that were used in the current study, and in Chapter 4 plasma Cu status was also decreased. However, it was not clear whether the reduction was due to the direct effect of thiomolybdate reducing Cu availability in the rumen or due to systemic effect of thiomolybdate affecting Cu metabolism after being absorbed from rumen. The predominate distribution of Cu, Mo and S in the solid phase and reducing Cu proportion in supernatant fraction following addition of Mo and S, therefore, support the proposed hypothesis of intraruminal formation Cu-thiomolybdate complex (Dick *et al*., 1975; Suttle, 1991; Gould and Kendall, 2011), and the role of the solid phase in reducing Cu availability in ruminants (Price and Chester, 1985; Allen and Gawthorne, 1987), and a reduction in Cu metabolism that was seen in Chapters 3 and 4.

5.5. Conclusion

The current study has shown that the forage sources grass silage, maize silage, dried grass pellets, and grass haylage when fermented *in vitro* in rumen fluid, differed in Cu, Mo, and S distribution among digesta fractions. The highest proportion of Cu in the supernatant fraction in grass haylage, followed by maize silage, then grass silage, while the lowest Cu proportion was in dried grass pellets. In addition, Cu and Mo were mainly found to be associated with the solid phase (strained solid, bacteria rich, and protozoa rich fractions) at the expense of the supernatant fraction, and inclusion of antagonists generally reduced Cu proportion in the supernatant. These findings support the hypothesis suggests that an intra-ruminal interaction between Cu, Mo and S leads to the formation insoluble Cu-thiomolybdates complex, which are poorly absorbed but greatly excreted and hence reduce Cu status.

Chapter 6 The effect of forage preservation and either supplemented without or with molybdenum and sulfur on rumen pH and on copper status in growing lambs

6.1. Introduction

The results from Chapters 3, and 4 indicated that a higher liver Cu status in growing lambs was associated with a lower rumen pH. Crosby *et al*. (2004) associated the lower liver Cu concentration in growing lambs housed on a straw-bedded floor compared with those housed on an expanded-metal with lower rumen acidity, which is caused by straw intake, increasing rumination and saliva production and resulted in a higher rumen pH (Ørskov, 1987). The higher rumen pH in turn encourages ciliate protozoa to reduce sulfur to sulfide, which in turn, may reduce dietary Cu availability (Suttle, 1979; Crosby *et al*., 2004; Spears *et al*., 2011). Equally, sulfide contributes to the mechanism by which Mo depresses Cu availability (Dick *et al*., 1975; Suttle, 2010). The rate of rumen sulfide production has been reported to be increased by continuous feeding compared with twice a day or every 4hrs (Suttle and Peter, 1985; Luo *et al*., 1996). In addition, the rate of eating fermentable carbohydrates, which reduces rumen pH and hence increases Cu availability due to an increase in sulfide absorption (Bray *et al*., 1975) or break down of thiomolybdates (Suttle, 1991).

In Chapter 5, the highest proportion of Cu and Mo and the highest pH was in the *in vitro* fermented GH in the rumen fluid compared with other forages. Copper absorption is suggested to be largely determined by synchronicity of release of Cu and its antagonists from feedstuffs into the rumen, the site of the CuxMoxS interactions (Suttle, 1983b; 1991). Therefore, preservation of forages may also affect the interaction between Cu and its antagonists.

There has been a trend for using whole crop cereal silages for feeding ruminants in recent years due to the similar cost of production relative to the grass silage and potential benefits in forage intake and subsequently animal performance (Keady *et al*., 2013). However, little information is available of the effect of whole crop wheat silage on the metabolism of Cu in ruminants. Therefore, the aims of this study were to evaluate effect of forage preservation on rumen pH and their interaction between Cu and its antagonists (Mo and S).

6.2. Materials and methods

6.2.1. Animal procedures

All procedure involving animals were carried out according to the UK Animals (Scientific Procedures) Act 1986 and were approved by Harper Adams University Ethic Committee.

6.2.2. Forage production

Two whole crop wheat (WCW) silages were made, the first one was WCW harvested at 400 g/kg treated with 4 L/tonne of additive (Whole Crop Gold, Biotal Limited, Cardiff, UK), rolled and sheeted to ferment and produce fermented WCW (FWCW). The second one used Santiago winter wheat and was harvested at 700 g/kg. It was treated with 4 kg/tonne urea and urease (Home n'Dry, Dugdale, Clitheroe, UK). It was also rolled and sheeted to produce alkalage or urea-treated WCW (UWCW). The third forage was a first cut grass silage from a predominately perennial ryegrass sward, which received additives (Biotal axcool gold, Waterford, Ireland, UK) and was ensiled in round bale.

6.2.3. Animals and experimental design

The study was carried out at Harper Adams University (at 7th of October 2015) using 68 castrated male Scottish Blackface growing lambs with an initial mean body weight of 22.96 kg (s.e.d; 0.309) over a period of 10 weeks. Lambs were brought from Perthshire, Scotland and were adapted for approximately 4 weeks prior to the start of the study and offered grass haylage at maintenance level. Eight representative lambs were slaughtered immediately prior to the start of the study at a commercial abattoir, and liver samples were collected and stored at -20 °C to serve as a baseline for liver Cu level (section 2.4.2). The remaining 60 lambs were blocked according to liveweight (LW) and randomly allocated to one of six treatments in a 3 x 2 factorial design with 10 lambs per treatment. The lambs were housed in a well-ventilated shed in individual pens and bedded on wood shavings. They had free access to fresh water.

6.2.4. Diets

Lambs were fed diets including *ad libitum* forage together with 300 g/day of a standard concentrate. The raw materials that were used to formulate the concentrate diet are presented in Table (6.1). The forages were either grass silage (GS), fermented WCW (FWCW) or urea treated WCW (UWCW). Two appropriate concentrates were formulated differently to allow for difference in forage CP levels (Table 6.2). One of the concentrates was fed with GS and UWCW forages, and other was fed with FWCW. Based on AFRC (1993) and the predicted intake of forage and concentrate (1 kg DM), the diet would provide sufficient Metabolisable Energy (ME) and Metabolisable Protein (MP) to meet requirements to growing at 200g/day. Predicted ME for GS, fermented WCW, and urea WCW diets was 11.3, 10.8, and 10.6 (MJ/kg DM) respectively.

Table 6.1. Raw material composition of the experimental concentrates (g/kg DM)

Ingredients, g/kg DM	Concentrate Diets[1]	
	GS and UWCW	FWCW
Barley	530	129
Sugar beet pulp	247	252
Soya bean meal	96	490
Molasses	58	59
Mins/vits[2]	69	70
Total	1000	1000

[1] GS and UWCW= concentrate fed with grass silage and urea treated WCW forages, FWCW= concentrate fed with fermented WCW.

[2] Mineral premix (25 kg/tonne) (Rumenco, Burton upon Trent, Staffordshire, UK). Major minerals (g/kg DM): Calcium, 185; Phosphorous, 20; Magnesium, 100; Sodium, 120; Chloride, 205; Trace elements (mg/kg DM); Iodine, 150; Cobalt, 90; Manganese, 3000; Zinc, 3000; Selenium (sodium selenite), 20. Vitamins; Vit A {E 672}, 320000 IU/kg; Vit D3 {E 671}, 100000 IU/kg. Vit E (all-rac-alpha-tocopheryl acetate) {3a700} 2000 mg/kg.

The individual components of the diet were analysed by ICP-MS (section 2.4.1), and the chemical composition of forages present in Table 6.2. Then, predicted trace element supply (Table 6.3) was calculated. Based on the equations of Suttle and MacLauchlan (1976), Mo and S was added to the diets to reduce Cu absorption by 50% from the availability of Cu in the diet. Levels added are presented in Table 6.3.

Table 6.2. Chemical composition of grass silage (GS), fermented WCW (FWCW), Urea WCW (UWCW).

items	Forage		
	GS	FWCW	UWCW
Chemical composition, g/kg DM			
DM, g/kg	237.6	365.6	600.2
CP,	149.8	97.2	164.6
EE,	34.9	17.8	12.6
NDF,	575.6	418.3	402.5
Ash,	98.5	48.6	40.1
ME, MJ/kg DM[1]	11.0	10.0	10.3
pH	4.3	3.9	8.7
Mineral composition, mg/kg DM			
Cu,	10.8	6.1	7.7
Mo,	2.27	1.47	1.22
S, g/kg DM	1.40	1.10	1.29
Fe,	140.1	97.9	106.1
Zn,	34.5	22.7	21.1
Mn,	71.6	38.8	19.5

ME= metabolisable energy of the forages was taken from AFRC (1993).

Table 6.3. The predicted mineral composition of the experimental diets.

Minerals, mg/kg DM	GS no added Mo and S	FWCW no added Mo and S	UWCW no added Mo and S	Additional levels of Mo and S
Cu,	9.74	7.69	7.05	
Mo,	1.71	2.49	2.45	3.5
S, g/kg DM	2.29	2.17	2.21	2

The Mo added was in the form ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (Fisher Scientific, Leicester, UK), and S was in the form of ammonium sulphate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA). The N content of the diets were balanced with Urea (Trouw Nutrition, Cheshire, UK). The feed grade urea (Trouw Nutrition, Northwich, Cheshire) was added (11.2 kg/tonne DM) to the fermented WCW unsupplemented with antagonists diet in order to balance the N level.

Lambs were allocated according to their liveweight to one of six dietary treatments (Table 6.4).

Table 6.4. Dietary treatments

Code	Treatment
GS-	Grass silage, no antagonists
GS+	Grass silage, supplemental Mo + S
FWCW-	Fermented WCW, no antagonists
FWCW+	Fermented WCW, supplemental Mo + S
UWCW-	Urea - treated WCW, no antagonists
UWCW+	Urea - treated WCW, supplemental Mo + S

Feed samples (forage and concentrates) were collected once weekly throughout the study. All feed samples were analysed for DM, Ash, CP, NDF, EE and mineral contents, as described in sections 2.1.1. to 2.1.5, and (section 2.4.1), respectively. The chemical composition of the experimental diets (forage and concentrate) of predicted forage intake to be 1 kg/d at DM basis are presented in Table 6.5.

6.2.5. Experimental routine

All lambs were offered feed twice a day at (08:30 and 16:30). Forages (GS, FWCW, or UWCW) were put into wooden troughs, and concentrates placed into plastic buckets. Feed refusals were collected twice a week (every Saturday and Thursday until the end of experiment) to estimate individual feed intake and feed conversion efficiency. At the end of the study, 54 out of 60 lambs were sent to a commercial abattoir for slaughtering. All lambs, including the eight representative lambs slaughtered on day 0, were slaughtered using electrical stunned. Livers were collected immediately after slaughter, weighed, and stored at -20°C for subsequent mineral content determination.

Table 6.5. Composition of concentrates and forages; grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) fed without (-) or with (+) added S and Mo[1].

Items	Forage			Concentrate			
	GS	FWCW	UWCW	GS- and UWCW-	GS+ and UWCW+	FWCW-	FWCW+
Chemical composition, g/kg DM							
DM, g/kg	227.3	340.1	627.4	829.7	828.2	833.8	835.6
CP,	155.7	106.1	146.1	142.1	176.7	340.5	348.1
EE,	30.9	12.9	13.6	11.2	11	11.2	11.1
NDF,	588.2	413.6	309.2	156.4	146.7	153.3	145
Ash,	115.1	53.4	44.4	128.3	112.4	132.1	130
Mineral composition, mg/kg DM							
Cu ,	11.54	6.86	6.74	11.15	11.26	11.26	11.52
Mo,	1.11	0.88	1.02	1.36	19.54	1.81	19.91
S ,g/kg	2.69	1.13	1.1	6	17.51	7.72	16.39
Fe,	140.5	97.9	106.2	346.5	344.1	338.6	335.4
Zn,	30.4	23.3	20.1	160.2	161.7	159.8	164.6
Mn,	84.7	54.4	43.4	110.9	106.3	109.4	111.4

[1] Dietary treatments GS-, FWCW-, and UWCW- without including Mo and S. Diets GS+, FWCW+, and UWCW+ were included Mo 3.5 mg/kg as ammonium molybdate and S 2 g/kg DM as ammonium sulfate.

6.2.5.1. Blood sample collection

Blood samples were collected by jugular vein puncture (section 2.2.) once a week on Thursday at 11:00 for plasma and serum ceruloplasmin analysis (sections 6.2.6). On weeks 0, 4, 8, and 10 an additional EDTA tube was collected for haematology analysis and an aliquot stored at -20°C for SOD analysis (section 6.2.6).

6.2.5.2. Liveweight determination

Lambs were weighed once a week on Wednesday at 11:00 using the standard operating procedure as described in section 2.3. Daily liveweight gain (DLWG) was calculated using regression analysis.

6.2.6. Blood analysis

Fresh blood samples after being collected were quickly analysed for haematocrit (Hct), haemoglobin concentration (Hb), red blood cell counts (RBC), and white blood cell counts (WBC) using a Vet Animal Blood Counter (section 2.2.1). Whole blood samples were analysed for SOD activity using a Cobas Mira Plus as described in section 2.2.3.1. Plasma samples were used to determine mineral concentrations (section 2.2.2). Serum samples were also analysed for ceruloplasmin activity (Cp) using a Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) (section 2.2.3.2).

6.2.7. Liver mineral concentrations

Liver samples were analysed for mineral concentrations using an ICP-MS as described in section 2.4.2. Whole liver minerals content was determined by multiplying liver mineral concentrations by liver weight and by liver DM. Liver minerals retention was determined by substracting whole liver mineral content of the initial slaughter group from final whole liver minerals content and dividing by days of the whole study period.

6.2.8. Rumen pH determination

Rumen fluid samples were collected immediately after slaughter of the lambs, put into 100 ml plastic pots and stored on ice prior to measuring pH within an hour after slaughtering (section 2.1.6).

6.2.9. Statistical analysis

Performance, plasma minerals, haematology, and enzyme activities were analysed by repeated-measures ANOVA as a 3x2 factorial randomised block design with the main effects of forage type (F), and antagonists (Ant.). Daily live weight gain (DLWG) was calculated by regression analysis and analysed by ANOVA. For plasma Cu and zinc concentrations, Ceruloplasmin activity, Cp:Pl-Cu ratio, SOD activity, Haematocrit, Haemoglobin concentration, and RBC counts week zero was used as a covariate. All statistical analysis were conducted using Genstat version 17.1 (Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Significance was set at $P < 0.05$ and trends at $P < 0.10$. Significant differences between means were tested using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

6.3. Results

6.3.1. Health observation

During the study, 6 lambs out of 60 were removed from the study as a result of losing more than 10% of the body weight due to Pneumonia. Of these three were supplemented with Mo and S. Removed lambs belonged to all dietary treatments, one lamb on grass silage supplemented antagonists, and fermented WCW unsupplemeted with antagonists treatment, two lambs on urea WCW supplemented without or with antagonists treatment.

6.3.2. Animal performance and intake

There was no forage x antagonist interaction (P>0.05) on weekly liveweight, DLWG, DMI, and FCE of the lambs throughout the study (Fig. 6.1 and Table 6.7). There was also no effect (P>0.05) of the antagonists on these parameters, except the concentrate DMI was lower (P<0.05) when Mo and S was supplemented compared with unsupplemented, with mean values of 0.23 and 0.24 kg/d respectively. Lambs fed urea WCW were heavier (P<0.05) from week 8, 9, and 10 compared with the lambs fed fermented WCW or GS forage (Table 6.6). Lambs offered urea WCW and fermented WCW had a higher (P<0.05) forage and total DMI compared to those offered GS. The urea WCW fed lambs also had a higher (P<0.001) DLWG and FCE compared with those fed fermented WCW or GS forages.

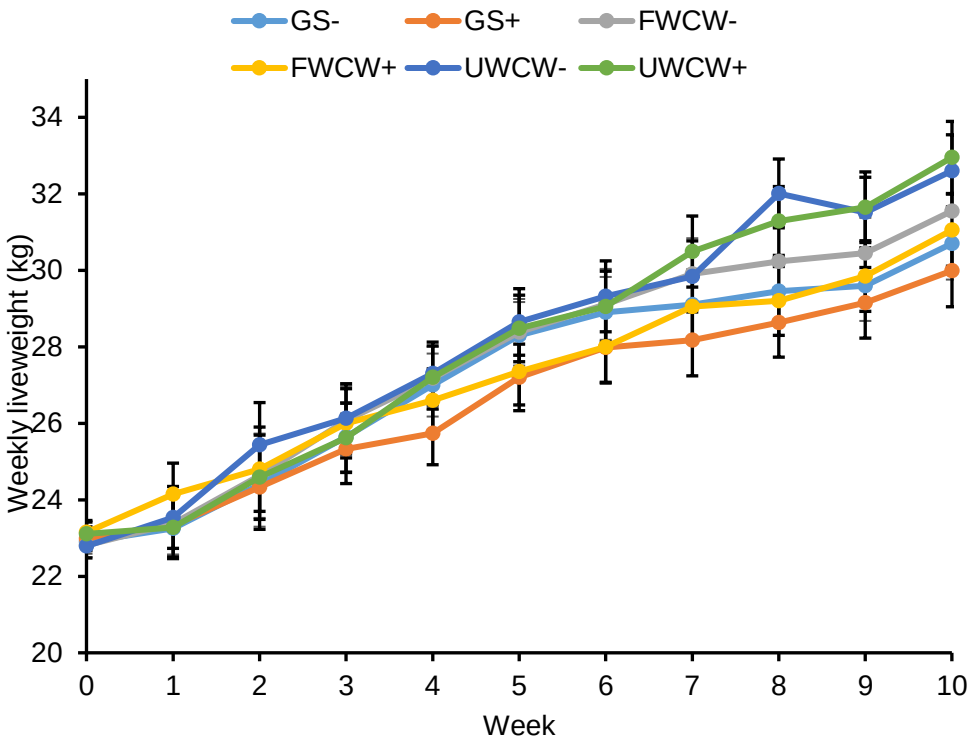


Figure 6.1. The effect of forage type grass silage (GS), fermented WCW (FWCW), and WCW (WCW) supplemented without (-) or with (+) Mo and S on weekly liveweight. Error bars indicate SED.

Table 6.6. Effects of forage type (grass silage (GS), fermented WCW (FWCW), and urea treated WCW (UWCW) on weekly lamb liveweight.

week[2]	Forages			Significance[1,3]	
	GS	FWCW	UWCW	s.e.d	P-value
0	22.9	23.0	22.9	0.22	0.983
1	23.3	23.8	23.4	0.57	0.695
2	24.4	24.7	25.0	0.78	0.707
3	25.5	26.0	25.9	0.64	0.688
4	26.4	26.9	27.3	0.58	0.323
5	27.8	27.9	28.6	0.62	0.366
6	28.4	28.6	29.2	0.66	0.478
7	28.6	29.5	30.2	0.66	0.078
8	29.0[a]	29.7[a]	31.7[b]	0.64	<.001
9	29.4[a]	30.2[a]	31.6[b]	0.65	<.006
10	30.4[a]	31.3[a]	32.8[b]	0.67	<.003

[1] s.e.d= standard error of difference.

[2 a,b] superscripts within rows indicate significant difference at (P<0.05).

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Table 6.7. Effects of forage type; grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S on the performance and rumen pH of growing lambs[1].

Items[2]	Treatment						Significance		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
Intake, kg/d										
Forage DMI,	0.44	0.42	0.52	0.50	0.52	0.50	0.035	0.003	0.185	0.994
Concentrate DMI,	0.25	0.23	0.23	0.24	0.25	0.23	0.007	0.359	0.021	0.192
Total DMI,	0.69	0.65	0.76	0.73	0.77	0.73	0.038	0.007	0.106	0.946
DLWG, kg/d	0.12	0.10	0.13	0.11	0.14	0.15	0.012	<.001	0.201	0.303
FCE[3]	0.17	0.16	0.17	0.15	0.19	0.21	0.015	0.002	0.599	0.150

[1] F= main effect of forages; A= antagonist, Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[2] DMI= total dry matter intake, DLWG= daily liveweight gain, FCE= feed conversion efficiency.

[3] FCE calculated as DLWG (kg/d) divided by DMI (kg/d).

6.3.3. Mineral intake

There was no forage x antagonist interaction (P>0.05) on Cu, Mo, Fe, Zn, or Mn intake (Table 6.8). However, there was a forage x antagonist interaction on S intake (P<0.05). The intake of S was higher in lambs fed grass silage supplemented with antagonists, followed by lambs fed fermented or urea WCW supplemented with antagonists, and the lambs fed grass silage and fermented whole crop with no added antagonists, and the lowest S intake in lambs fed urea WCW with no added antagonists.

Lambs offered grass silage had a higher (P<0.001) Cu (7.63 mg of Cu/d) and S (3.91 g of S/d) intake compared with the lambs fed fermented WCW (6.16 mg of Cu/d and 3.40 g of S/d) or urea WCW (6.15 mg of Cu/d and 3.37 g of S/d) (s.e.d, 0.225 for Cu and 0.10 for S). Lambs fed fermented WCW had a lower Fe intake (128.7 mg/d) compared with those fed grass silage (142.5 mg/d) or urea WCW (137.4 mg/d) (s.e.d, 3.78). The highest Mn intake was in lambs fed grass silage (62.3 mg/d), followed by lambs fed fermented WCW (53.6 mg/d), while lambs fed urea WCW had the lowest Mn intake (48.5 mg/d) (s.e.d, 1.69).

There was no effect of addition of antagonists on Cu or Zn intake (P>0.05). The addition of antagonists into the diet increased Mo and S intake compared with lambs fed diets with no added antagonists. Lambs fed diets unsupplemented with antagonists had a higher (P<0.05) Fe and Mn intake compared with those fed diet supplemented with antagonists.

Table 6.8. Minerals intake in growing lambs fed diets containing grass silage (GS), fermented WCW, or urea WCW (UWCW) supplemented without (-) or with Mo and S.

Minerals, mg/d	Treatment							Significance[2]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
Cu,	7.9	7.4	6.2	6.1	6.3	6.0	0.32	<.001	0.102	0.728
Mo,	0.83	4.96	0.87	5.11	0.89	5.09	0.139	0.519	<.001	0.868
S, g/d	2.7^{b}	5.1^{d}	2.4^{b}	4.4^{c}	2.1^{a}	4.7^{c}	0.14	<.001	<.001	0.017
Fe,	147.5	137.5	130.2	127.2	141.5	133.3	5.34	0.003	0.027	0.641
Zn,	52.8	49.8	49.6	50.2	50.3	47.9	1.65	0.162	0.100	0.270
Mn,	64.9	59.7	54.1	53.1	50.4	46.5	2.39	<.001	0.020	0.454

[1] F= main effect of forages; A= antagonist, Int.= interaction between forage type and antagonists. s.e.d= standard error of difference.

[2] [a,b,c,d] Means within a row with different superscripts are significantly different (P<0.05).

6.3.4. Rumen pH

There was no forage x antagonist interaction (P>0.05) on rumen pH of lambs (Fig. 6.2). There was also no effect (P>0.05) of Cu antagonists on rumen pH of the lambs. However, there was a trend (P=0.059) for a higher rumen pH in lambs fed GS forage (6.38) compared with those fed urea WCW (6.22) or fermented WCW (6.21) (s.e.d, 0.077).

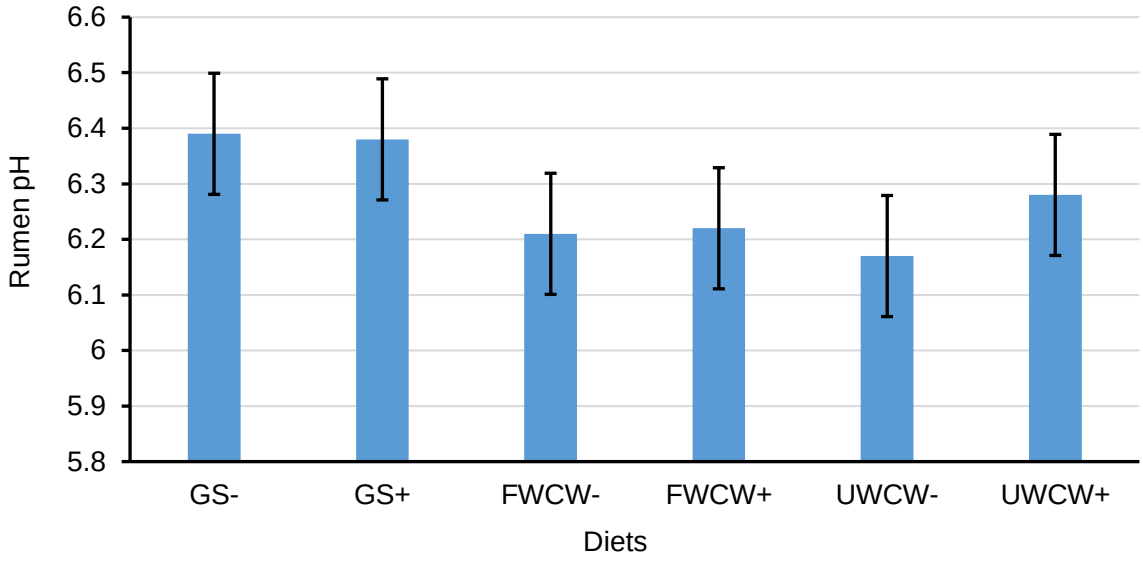


Figure 6.2. Rumen pH of the growing lambs fed forages containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S. Error bars indicate SED.

6.3.5. Liver mineral status

6.3.5.1. Liver mineral concentrations

The mean liver concentration of Cu, Mo, Fe, Zn and Mn of the representative lambs slaughtered at the beginning of the experiment are presented in Table 6.9.

There was no forage x antagonist interaction (P>0.05) on liver Cu, Mo, Zn, or Mn concentration (Table 6.10). However, there was a forage x antagonist interaction on liver Fe concentration (P<0.05). Lambs offered urea WCW supplemented without or with antagonists had a lower liver Fe concentration compared with lambs fed grass silage supplemented with antagonists or grass silage or fermented WCW unsupplemented with antagonists, with fermented WCW supplemented with antagonists being intermediate.

Lambs fed urea WCW or grass silage had a higher (P<0.001) liver Cu concentration compared with lambs fed fermented WCW, with mean values of 188.5, 171.6, and 119.0 mg/kg DM respectively (s.e.d, 17.58). The urea WCW fed lambs had a higher (P<0.05) liver Mo concentration (4.44 mg/kg DM) compared with the grass silage fed lambs (3.43 mg/kg DM), with lambs fed fermented WCW being intermediate (4.0 mg/kg DM) (s.e.d, 0.325). Liver Fe concentration was lower (P<0.001) in the lambs offered urea WCW (374 mg/kg DM) compared with those offered grass silage (512 mg/kg DM) or fermented WCW (468 mg/kg DM) (s.e.d, 30.3). There was no difference between forage source on lamb liver Zn and Mn concentrations (P>0.05).

Lambs offered a diet supplemented with Mo and S had a lower liver Cu and Zn concentration compared with the lambs not receiving antagonists (P<0.05). In contrast, additional antagonists resulted in an increase in the liver Mo concentration compared with those fed diet unsupplemented with antagonists. Adding Cu antagonists had not an effect on liver Fe and Mn concentrations (P>0.05).

Table 6.9. The initial liver mineral status of growing lambs.

Liver minerals, mg/kg DM	Concentration (mg/kg DM)	Standard Deviation
Cu,	341.8	± 124.4
Mo,	2.5	± 0.7
Fe,	571.1	± 49.2
Zn,	100.3	± 8.3
Mn,	87.7	± 49.2

Table 6.10. Liver minerals concentration of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S.

Minerals, mg/kg DM	Treatment							Significance[1]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
Cu,	208.4	134.7	131.2	106.8	237.3	139.8	24.87	<.001	<.001	0.119
Mo,	2.47	4.39	3.40	4.59	3.50	5.38	0.460	0.013	<.001	0.453
Fe,	538^{d}	486^{bcd}	503^{cd}	433^{bc}	336^{a}	412^{ab}	42.9	<.001	0.537	0.041
Zn,	106.9	92.6	120.8	95.7	110.5	104.5	9.03	0.349	0.006	0.332
Mn,	52.7	42.5	56.0	41.7	42.9	50.5	10.75	0.961	0.366	0.318

[1]F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] [a,b,c,d] Means within a row with different superscripts are significantly different (P<0.05).

6.3.5.2. Whole liver mineral content

There was no forage x antagonist interaction (P>0.05) on whole liver Cu, Mo, Zn, or Mn content (Table 6.11). There was a forage x antagonist interaction (P<0.05) on whole liver Fe content, with the lowest content in lambs offered urea WCW unsupplemented with antagonists compared with the lambs fed any of the other diets.

Lambs fed fermented WCW had a lower (P<0.001) liver Cu content compared with lambs fed urea WCW or grass silage. Liver Mo and Zn content were lower in lambs offered grass silage than those offered urea or fermented WCW (P<0.05). The urea WCW resulted in a lower liver Fe content compared with the lambs fed grass silage or fermented WCW (P<0.001). There was no effect of forage type on liver Mn content (P>0.05).

There was no effect (P>0.05) of antagonists on whole liver Fe, Zn, and Mn content. However, lambs fed diets supplemented with Mo and S had a higher (P<0.001) whole liver Cu content compared with the lambs not receiving Mo and S (26.1 and 17.7 mg/liver; s.e.d, 1.91 respectively). The addition of antagonists increased (P<0.001) liver Mo concentration compared with lambs fed diet no added antagonists, with mean values of 0.68 and 0.43 mg/liver (s.e.d, 0.041) respectively.

Table 6.11. Whole liver mineral content of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S[1].

Minerals, mg/liver	Treatment							Significance[2]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
Cu,	26.9	18.1	18.1	15.7	33.5	19.3	3.30	<.001	<.001	0.051
Mo,	0.32	0.58	0.48	0.68	0.49	0.79	0.071	0.002	<.001	0.593
Fe,	69.7[b]	65.0[b]	68.7[b]	62.6[b]	46.7[a]	59.1[b]	5.66	0.001	0.876	0.047
Zn,	13.8	12.4	16.8	14.4	15.6	15.3	1.51	0.043	0.127	0.621
Mn,	6.9	5.7	7.9	6.2	6.0	7.2	1.57	0.787	0.546	0.383

[1] whole liver minerals content = final whole liver weight x final liver DM x final liver Cu concentration (mg/kg DM).

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

6.3.5.3. Whole liver mineral retention

There was no forage x antagonist interaction (P>0.05) on whole liver retention for Mo, Fe, Zn, and Mn (Table 6.12). However, there was a forage type x antagonist interaction (P<0.05) on the whole liver Cu retention. Liver Cu retention in lambs fed urea WCW and grass silage was reduced (P<0.05) by addition of antagonists, but in lambs on fermented WCW addition of antagonists had no an effect (P>0.05) on liver Cu retention.

There was no effect of forage type on liver Zn and Mn retentions (P>0.05). However, liver Cu retention in lambs on fermented WCW was lower (P>0.05) compared with lambs on urea WCW or grass silage. Liver Mo retention was also affected by forage type (P<0.05). Liver Mo retention was higher in lambs fed urea WCW, intermediate in lambs fed fermented WCW and the lowest in lambs fed grass silage. Lambs offered urea WCW had a lower liver Fe retention than lambs offered grass silage or fermented WCW (P<0.001). Liver Zn and Mn retention was not affected by forage type (P>0.05).

There was no effect of antagonists on liver Fe, Zn, and Mn retention (P>0.05). However, compared with the initial group, the rate of reduction in liver Cu retention was higher (P<0.01) in lambs fed diets supplemented with antagonists compared with lambs fed diets with no added antagonists, with mean values of 0.022 and 0.09 mg/day (s.e.d, 0.029) respectively. Lambs fed diets supplemented with antagonists had a higher liver Mo retention compared with lambs fed diets unsupplemented with antagonists, with mean values of 5.87 and 2.51 µg/day (s.e.d,0.698) respectively.

Table 6.12. Whole liver mineral retention of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S[1].

Minerals, mg/day	Treatment							Significance[2]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
Cu,	-0.08[b]	-0.20[a]	-0.21[a]	-0.24[a]	0.02[b]	-0.22[a]	0.05	0.004	<.001	0.019
Mo,µg/day	0.95	4.82	3.02	6.15	3.55	6.65	1.21	0.040	<.001	0.880
Fe,	0.19	0.12	0.16	0.09	-0.14	-0.06	0.10	<.001	0.730	0.480
Zn,	0.06	0.04	0.09	0.07	0.08	0.05	0.03	0.190	0.080	0.950
Mn,	-0.36	-0.38	-0.35	-0.37	-0.37	-0.36	0.02	0.770	0.420	0.660

[1] liver minerals retention were calculation by substrate whole liver minerals content at day zero from final whole liver Cu content divided by whole study period (days).
[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

6.3.6. The mean of plasma mineral profile, Cu-mediated enzymes, and haematology profile

No effect (P>0.05) was observed of dietary treatment on the mean plasma Cu and Zn concentrations (Table 6.13). There was an effect (*P*<0.05) of forage type on the mean Pl-Mo concentration, which was higher in lambs fed urea WCW, intermediate in lambs fed fermented WCW, and lower in those fed grass silage. Lambs fed diet supplemented with Mo and S had a higher (*P*<0.001) mean Pl-Mo concentration compared with those fed diet unsupplemnted with antagonists. There was no effect (*P*>0.05) of forage x antagonist interaction, or the addition of antagonists on the mean Pl-Fe concentration. However, there was an effect (*P*<0.05) of forage type on the mean Pl-Fe concentration, which was higher in lambs fed grass silage, intermediate in lambs fed fermented WCW, and lower in lambs fed urea WCW. No effect (*P*>0.05) was observed of dietary treatment on the mean Cp activity. There was also no effect (*P*>0.05) of forage x antagonists interaction, or forage type on the mean Cp:Pl-Cu ratio, but lambs fed diets supplemented with antagonists had a lower (*P*<0.05) mean Cp:Pl-Cu ratio compared with those fed diets unsupplemented with antagonists. There was a forage x antagonist interaction (*P*<0.05) on the mean of Hb concentration. Compared with unsupplemented antagonists, additional Mo and S decreased the mean Hb concentration in lambs fed fermented WCW, but lambs fed grass silage or urea WCW were not affected by the addition of antagonists. The mean Hct, RBC, and WBC were not afftected by dietary treatment (*P*>0.05).

Table 4.13. Effect of forage type grass haylage (GH) and maize silage (MS) fed without (-) or with (+) added Mo and S on mean indicators of blood Cu status over the study period of lambs [1].

Items	Treatments						Significance			
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
Cu, µmol/L	16.3	15.9	16.3	17.2	16.9	16.6	1.10	0.323	0.897	0.336
Mo, µmol/L	0.08	0.71	0.10	1.07	0.21	1.20	0.173	0.002	<.001	0.061
Fe, µmol/L	47.0	46.2	44.1	42.9	42.3	40.2	4.89	0.030	0.385	0.946
Zn, µmol/L	8.0	7.9	8.4	7.7	8.4	7.9	0.52	0.784	0.059	0.542
Cp, mg/dL	17.5	15.9	17.0	17.2	19.1	16.0	2.99	0.770	0.107	0.388
Cp:Pl-Cu	1.08	0.98	1.07	1.00	1.22	0.95	0.152	0.663	<.001	0.098
Hct, %	33.6	32.7	35.0	33.5	34.3	34.3	1.45	0.193	0.168	0.512
Hb, g/dL	11.3	11.3	11.9	10.9	11.7	11.5	0.44	0.407	0.031	0.047
RBC, $10^6/mm^3$	12.2	12.0	12.5	11.8	12.3	11.9	0.51	0.990	0.104	0.761
WBC, $10^3/mm^3$	9.5	8.6	10.6	8.9	9.0	8.6	1.07	0.299	0.059	0.592

[1] week 0 values were used as a covariate where appropriate.

[2] Hct- haematocrit; Hb= haemoglobin; RBC= red blood cells; WBC= white blood cells.

[3] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d = standard error of difference.

6.3.7. Plasma mineral concentrations

6.3.7.1. Plasma copper concentration

Repeated measures analysis indicated that there was an effect ($P<0.001$) of time on Pl-Cu concentration, with plasma concentrations declining over the period of the study (Table 6.14). However, there was no effect of time x treatment on Pl-Cu concentration ($P>0.05$).

There was no effect ($P>0.05$) of dietary treatment on Pl-Cu concentration throughout the study

Table 6.14. Plasma copper concentration of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

week	Treatment[2]							Significance[3]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
0	16.6	18.0	16.3	17.9	17.5	19.5	1.57	--	--	--
2	18.8	18.9	19.3	19.9	18.4	19.9	1.24	0.644	0.322	0.707
4	16.3	15.5	16.3	17.1	17.0	16.9	1.01	0.326	0.899	0.517
6	14.3	13.7	14.4	15.6	15.0	15.0	1.23	0.442	0.798	0.595
8	15.0	14.3	15.2	16.6	16.9	15.0	1.08	0.126	0.304	0.050
10	15.7	15.0	14.9	16.5	15.6	15.1	1.10	0.854	0.836	0.275

[1] week zero calculated as a covariate.

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.377	<.001
Time x Forage effect	0.503	0.914
Time x Antagonist effect	0.424	0.156
Time x Forage x Antagonist effect	0.778	0.735

6.3.7.2. Plasma molybdenum concentration

There was an effect (P<0.001) of time on Pl-Mo concentration (Table 6.15). There were also a time x forage interaction, and time x antagonist interaction on Pl-Mo concentration (P<0.05). There was a trend for interaction between time and forage type and antagonist on Pl-Mo concentration (P<0.1).

During week 8 and 10, there was a forage x antagonist interaction (P<0.001) on Pl-Mo concentration, where the increase in Pl-Mo concentrations by inclusion of Mo and S in the urea WCW and fermented WCW fed lambs was greater compared with the GS fed lambs. At week 4 until week 10, the urea WCW or fermented WCW fed lambs had a higher (P<0.05) Pl-Mo concentration compared with the GS fed lambs. At week 2 until week 10, the inclusion of Mo and S resulted in an increase in Pl-Mo concentrations compared with the lambs not receiving antagonists (P<0.001).

6.3.7.3. Plasma iron concentration

There was an effect (P<0.001) of time on Pl-Fe concentration, with levels decreasing over a period of the study (Table 6.16). There was also a time x forage interaction on Pl-Fe concentration (P<0.05). However, there were no time x forage x antagonist interaction or time x antagonist interaction on Pl-Fe concentrations (P>0.05).

There was no effect (P>0.05) of forage x antagonist interaction on Pl-Fe concentration at any weekly time points. Similarly, there was no effect of antagonists on Pl-Fe concentration (P>0.05). However, during week 6, lambs offered urea WCW had a lower (P<0.05) Pl-Fe concentration compared with lambs offered grass silage or fermented WCW. At week 8 and 10, lambs offered fermented or urea WCW had a lower Pl-Fe concentration compared with lambs fed grass silage (P<0.05).

Table 6.15. Plasma molybdenum concentration of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

Week	Treatment[2]						Significance[3]			
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW	s.e.d	F	A	Int
0	0.17	0.23	0.14	0.15	0.19	0.13	0.096	0.695	0.980	0.710
2	0.04	1.04	0.08	0.95	0.18	1.21	0.183	0.332	<.001	0.788
4	0.05	1.09	0.09	1.68	0.19	1.95	0.251	0.023	<.001	0.113
6	0.06	0.77	0.09	1.37	0.27	1.40	0.172	0.004	<.001	0.065
8	0.07^{a}	0.62^{b}	0.10^{a}	1.25^{c}	0.22^{a}	1.41^{c}	0.168	0.001	<.001	0.016
10	0.06^{a}	0.51^{b}	0.13^{a}	1.06^{c}	0.23^{a}	1.07^{c}	0.137	0.001	<.001	0.041

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[2] ^{a,b,c} Means within a row with different superscripts are significantly different (P<0.05).

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.058	<.001
Time x Forage effect	0.081	0.007
Time x Antagonist effect	0.066	0.001
Time x Forage x Antagonist effect	0.122	0.074

Table 6.16. Plasma iron concentration of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S (µmol/L) [1].

Week	Treatment							Significance[2]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW	s.e.d	F	A	Int
0	73.3	45.9	44.7	47.0	49.1	52.1	15.93	0.469	0.425	0.315
2	47.8	42.7	44.3	36.0	40.5	39.5	7.29	0.517	0.261	0.783
4	41.3	41.5	43.8	40.6	44.4	39.0	4.61	0.972	0.311	0.696
6	60.6	57.9	54.2	54.5	41.9	43.5	5.53	<.001	0.928	0.852
8	44.7	40.5	36.2	38.8	35.7	37.4	3.19	0.024	0.976	0.276
10	45.6	47.7	40.6	40.6	40.7	36.1	3.86	0.012	0.713	0.451

[1] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[2] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	1.81	<.001
Time x Forage effect	1.93	0.042
Time x Antagonist effect	1.58	0.922
Time x Forage x Antagonist effect	3.46	0.612

6.3.7.4. Plasma zinc concentration

There was an effect (P<0.001) of time on Pl-Zn concentration, with concentrations increasing over a period of the study (Table 6.17). There was also a time x treatment interaction on Pl-Zn concentration (P<0.05).

There was no forage x antagonist interaction on Pl-Zn concentration throughout the study (P>0.05). There was also no effect of forage type on Pl-Zn concentration, except at week 4. At week 4, lambs fed urea WCW had a higher (P<0.05) Pl-Zn concentrations compared with lambs fed fermented WCW or grass silage, with mean values of 8.85, 7.93, and 7.91 µmol/L (s.e.d, 0.456) respectively. At week 6, the Mo and S fed lambs had a lower (P<0.05) Pl-Zn concentration compared with lambs fed diets unsupplemented with Mo and S, with a mean values of 8.3 and 9.3 µmol/L (s.e.d, 410) respectively.

Table 6.17. Plasma zinc concentration of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S (µmol/L)[1].

Week	Treatment[2]							Significance[3]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW	s.e.d	F	A	Int
0	7.6	6.3	7.3	6.7	7.5	6.8	0.53	--	--	--
2	7.4	7.9	7.9	7.8	7.7	7.3	0.55	0.631	0.907	0.490
4	7.9	7.9	8.7	7.2	9.0	8.7	0.60	0.047	0.090	0.194
6	8.8	8.6	9.1	8.2	9.8	8.1	0.66	0.801	0.017	0.264
8	9.2	8.2	9.0	8.5	8.7	7.9	0.62	0.540	0.053	0.826
10	7.8	7.9	8.5	7.3	8.3	8.3	0.50	0.307	0.229	0.148

[1] week zero used as a covariate

[2] F= main effect of forage type; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.16	<.001
Time x Forage effect	0.27	0.046
Time x Antagonist effect	0.25	0.022
Time x Forage x Antagonist effect	0.37	0.046

6.3.8. Ceruloplasmin activity

There was an effect (P<0.001) of time on Cp activity, which was increasing over a study period (Table 6.18). There was also a time x antagonist interaction on Cp activity (P<0.05). However, there was no time x forage x antagonist interaction or time x forage interaction on Cp activity (P>0.05).

There was no forage x antagonist interaction on Cp activity throughout the study (P>0.05). There was also no effect of forage type on Cp activity (P>0.05). During week 4, lambs fed diets supplemented with Mo and S had a lower (P<0.05) Cp activity compared with those not receiving Mo and S, with mean values of 13.5 and 19.8 (s.e.d, 1.68) respectively.

6.3.9. Ceruloplasmin to plasma copper ratio (Cp:Pl-Cu ratio)

There was an effect (P<0.001) of time on Cp:Pl-Cu ratio (Table 6.19). There was also a time x antagonist interaction on Cp:Pl-Cu ratio (P<0.05). However, there was no (P>0.05) time x forage x antagonist interaction, or time x forage interaction on the Cp:Pl-Cu ratio.

There was no effect of forage type on Cp:Pl-Cu ratio of the lambs at any weekly time points (P>0.05), exept at week 10, there was a forage x antagonist interaction on Cp:Pl-Cu ratio. At week 10, there was a forage x antagonist interaction (P=0.002) on Cp:Pl-Cu ratio, when the additon of antagonists reduced Cp:Pl-Cu ratio in lambs on urea WCW compared with lambs on urea WCW unsupplemented with antagonists, wherease there was no difference (P>0.05) in Cp:Pl-Cu ratio between lambs fed grass silage or fermented WCW unsupplemented or supplemented with antagonists. During week 4, lambs fed diets unsupplemented with antagonists had a higher Cp:Pl-Cu ratio compared with those fed diets supplemented with Mo and S, with mean values of 1.2 and 0.8 (s.e.d, 0.10) respectively. There was no effect of forage type on Cp:Pl-Cu ratio of the lambs at any weekly time points (P>0.05)

Table 6.18. Ceruloplasmin activity of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) added S and Mo and S (mg/dL)[1].

week	Treatment[2]							Significance[3]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
0	9.3	11.2	9.6	10.7	12.6	11.4	1.34	--	--	--
2	19.7	18.1	17.4	19.1	18.5	17.0	3.29	0.882	0.811	0.716
4	17.1	13.7	20.2	14.4	21.9	12.2	2.95	0.593	<.001	0.330
6	20.8	18.8	20.0	22.1	19.1	21.6	3.74	0.898	0.675	0.647
8	19.1	18.6	17.8	19.5	23.3	20.0	3.65	0.487	0.724	0.631
10	10.7	10.2	9.8	10.8	12.6	9.2	1.27	0.838	0.161	0.057

[1] week zero used as a covariate

[2] F= main effect of forages; A = main effect of antagonists (Mo and S); Int. = interaction between forages and antagonists. s.e.d= standard error of difference.

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	1.14	<.001
Time x Forage effect	1.70	0.855
Time x Antagonist effect	0.93	0.034
Time x Forage x Antagonist effect	2.11	0.725

Table 6.19. The ratio of Cp:Pl- Cu of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) Mo and S[1].

Week	Treatment[2]							Significance[4]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int.
0	0.56	0.61	0.61	0.60	0.73	0.61	0.069	--	--	--
2	1.02	0.93	0.88	0.94	1.05	0.88	0.140	0.807	0.402	0.482
4	1.04	0.80	1.25	0.84	1.32	0.70	0.168	0.594	<.001	0.293
6	1.43	1.27	1.44	1.41	1.37	1.28	0.151	0.612	0.281	0.848
8	1.25	1.31	1.14	1.16	1.51	1.29	0.216	0.300	0.726	0.630
10[3]	0.67[a]	0.67[a]	0.65[a]	0.65[a]	0.84[b]	0.59[a]	0.054	0.355	0.011	0.002

[1] week zero used as a covariate.

[2] F= main effect of forages; A= antagonists; Int. = interaction between forages and antagonist. s.e.d= standard error of difference.

[3] [a,b] Means within a row with different superscripts are significantly different (P<0.05).

[4] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.062	<.001
Time x Forage effect	0.050	0.367
Time x Antagonist effect	0.039	0.019
Time x Forage x Antagonist effect	0.107	0.612

6.3.10. Superoxide dismutase activity

Week 0 was used as a covariate. At week 10, there was no effect (P>0.05) of dietary treatment on SOD activity (Fig. 6.3).

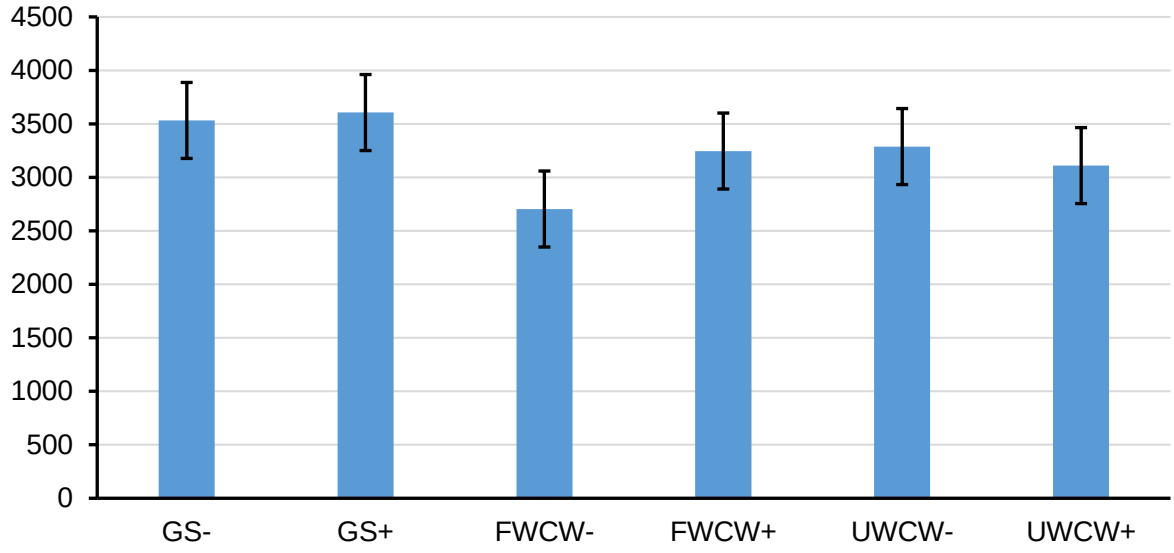


Figure 6.3. superoxide dismutase activity of growing lambs fed diets containing grass silage (GS), fermented whole crop wheat (FWCW) or urea-treated whole crop wheat (UWCW) supplemented without (-) or with (+) Mo and S. Error bars indicate SED.

6.3.11. Haematology parameters

6.3.11.1. Haematocrit

There was an effect (P<0.001) of time on haematocrit (%), with values gradually increasing in all treatments (Table 6.20). However, there was no time x treatment interaction on Hct (P>0.05).

There was no effect (P>0.05) of dietary treatment on Hct at any weekly time points.

6.3.11.2. Haemoglobin concentration

There was an effect (P<.001) of time on Hb concentration, with concentrations increasing in all treatments with time (Table 6.21). There was no effect of time x treatment interaction on Hb concentration (P>0.05).

There was no (P>0.05) forage x antagonist interaction on Hb concentration throughput the study. However, there was an effect of forage type on blood Hb concentration at week 10; the concentration of Hb was higher in lambs fed urea WCW, intermediate in the fermented WCW fed lambs and lowest in lambs fed grass silage, with mean values of 12.11, 11.46, and 11.29 g/dL (s.e.d, 0.343) respectively. At week 10, lambs fed diets that were unsupplemented with Mo and S had a higher (P=0.003) blood Hb concentration compared with lambs not receiving antagonists, with mean values of 12.1 and 11.2 g/dL (s.e.d, 0.30) respectively.

Table 6.20. Haematocrit (Hct%) of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) Mo and S[1].

week	Treatment[2]							Significance[3]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
0	33.58	35.26	35.66	33.79	35.73	33.24	1.367	--	--	--
4	30.57	29.13	33.57	31.12	32.39	32.32	2.003	0.132	0.254	0.696
10	35.61	34.46	37	34.71	35.83	36.02	1.495	0.641	0.212	0.496

[1] week zero used as a covariate.

[2] F= main effect of forages; A= antagonists; Int. = interaction between forages and antagonist. s.e.d= standard error of difference.

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.531	<.001
Time x Forage effect	0.694	0.375
Time x Antagonist effect	0.576	0.470
Time x Forage x Antagonist effect	1.023	0.831

Table 6.21. Haemoglobin concentration (g/dL) of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) Mo and S[1].

week	Treatment[2]							Significance[3]		
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
0	11.78	11.58	11.86	11.65	12.38	10.96	0.454	--	--	--
4	10.81	11.17	11.67	10.5	10.97	11.03	0.548	0.964	0.404	0.113
10	11.5	11.08	12.4	10.51	12.35	11.88	0.498	0.049	0.003	0.066

[1] week zero used as a covariate.

[2] F= main effect of forages; A= antagonists Int. = interaction between forages and antagonist. s.e.d= standard error of difference.

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.157	<.001
Time x Forage effect	0.212	0.121
Time x Antagonist effect	0.185	0.120
Time x Forage x Antagonist effect	0.307	0.083

6.3.11.3. Red blood cell counts

There was an effect (P<0.001) of time on red blood cell counts, with the values increasing across all treatments (Table 6.22). However, there was no time x treatment interaction on RBC counts (P>0.05).

Red blood cell counts were not affected (P>0.05) by dietary treatment at any weekly time points.

6.3.11.4. White blood cell counts

There was an effect (P<0.001) of time on WBC counts, with values gradually increased with time in all treatments (Table 6.23). There was no effect of time x treatment interaction on WBC counts (P>0.05).

There was no effect (P>0.05) of forage x antagonist interaction on WBC counts in the lambs throughout the study. Likewise, there was no effect of forage type on WBC counts at any weekly time points (P>0.05). However, at week 4, lambs fed diet supplemented with antagonists had a lower WBC counts compared with lambs fed diet unsupplemnted with antagonists, with mean values of 7.9 and 9.2 $10^3/mm^3$ (s.e.d,0.55) respectively.

Table 6.22. Red blood cell counts of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) Mo and S (10^6/mm^3)[1].

Week	Treatment[2]						Significance[3]			
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
0	12.84	13.16	12.86	12.65	12.74	12.48	0.425	--	--	--
4	11.30	10.85	11.76	11.03	11.78	11.19	0.619	0.615	0.104	0.951
8	12.47	12.04	12.65	11.83	12.48	12.16	0.448	0.960	0.051	0.706

[1] week zero used as a covariate.

[2] F= main effect of forages; A= antagonists; Int. = interaction between forages and antagonist. s.e.d= standard error of difference.

[3] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.150	<.001
Time x Forage effect	0.294	0.511
Time x Antagonist effect	0.240	0.922
Time x Forage x Antagonist effect	0.362	0.994

Table 6.23. White blood cell counts of growing lambs fed diets containing grass silage (GS), fermented WCW (FWCW) or urea WCW (UWCW) supplemented without (-) or with (+) Mo and S ($10^3/mm^3$)[1].

Week	Treatment						Significance[2]			
	GS-	GS+	FWCW-	FWCW+	UWCW-	UWCW+	s.e.d	F	A	Int
0	10.35	10.39	11.62	9.85	9.26	9.52	1.090	0.212	0.440	0.361
4	9.14	7.39	9.65	8.66	8.69	7.61	0.954	0.280	0.026	0.828
8	9.09	8.02	10.57	8.29	9.06	8.72	1.155	0.563	0.073	0.489

[1] F= main effect of forages; A= antagonists; Int. = interaction between forages and antagonist. s.e.d= standard error of difference.

[2] Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage, time x antagonist, or time x forage x antagonist interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.302	<.001
Time x Forage effect	0.625	0.558
Time x Antagonist effect	0.510	0.259
Time x Forage x Antagonist effect	0.756	0.440

6.4. Discussion

The pH value of whole crop forages can give a good indication of its method of preservation (Adamson and Reeve, 1992). The WCW that was treated with urea was well preserved, as reflected by the pH value, which was above 8.0. It has been suggested by Adogla-Bessa *et al*. (1999) that a pH value above 8.0 indicates successful promotion of alkaline conditions, limiting the proliferation of fungi and saccharolytic clostridia, which often results in aerobic spoilage and DM loses (Adogla-Bessa *et al*., 1999). Similarly, the low pH value of pH 3.9 in fermented WCW shows the successful conversion of water soluble carbohydrates into organic acids, particularly lactic acid, which eliminates enzyme activity and undesirable microorganisms in forage during ensiling (Merry *et al*., 2000). It is therefore likely that in the current study fermented WCW and urea WCW were well preserved. The grass silage used in this study seemed to be also well preserved, with a low pH 4.2 (Bond, 2006).

The results of the current study demonstrated that DMI in growing lambs was higher when fed on either urea WCW or fermented WCW than GS, which may be due to the higher forage DM in WCW compared GS, as the high intake of WCW in cows has been suggested to possibly be associated with forage DM (Keady *et al*., 2007). Previous work also has shown that feeding WCW generally increased DMI compared to GS in cows (Leaver and Hill, 1995; Sinclair *et al*., 2003; Owens *et al*., 2009), or when it is partially replaced with fermented or urea treated WCW (Bond, 2006; Burke *et al*., 2007). The lack of a difference in DMI between lambs offered fermented WCW or urea WCW in the present study is consistent with the results of Leaver and Hill (1995), Phipps *et al*. (1995) and Walsh *et al*. (2008) who reported no significant difference in DMI between animals fed fermented WCW or urea WCW. The similarity in DMI between fermented WCW and urea WCW had been attributed to their similar chop length (Walsh *et al*., 2008), which was also similar in the current study.

The higher intake in the urea WCW fed lambs is also reflected in a higher weight gain compared with the lambs fed GS, possibly due to the greater energy intake as a result of the greater DMI (Murphy *et al*., 2004; Walsh *et al*., 2008). The higher weight gain in urea WCW compared with GS is in agreement with the results of with the result of Murphy *et al*. (2004), Burke *et al*. (2007) and Walsh *et al*. (2008) who reported that liveweight gain was significantly increased when animals were offered processed or urea WCW compared to GS. The higher weight gain in growing lambs fed urea WCW in the current study compared with those fed fermented WCW was despite having a similar DMI which may be related to an enhancement in synchronisation of supply of additional volatile N from urea in the urea

WCW, and the fermentable energy in WCW, resulting in enhanced microbial growth (Adogla-Bessa *et al*., 1993; Sinclair *et al*., 1993), and animal performance (Bond, 2006).

Performance characteristics such as intake and liveweight gain of the growing lambs were not affected by Cu antagonists. These results are similar to the results of Chapters 3 and 4. However, in the current study, 6 lambs were removed from the study due to losing weight as a result of being affected by pneumonia. The removed lambs were from all dietary treatments, therefore, the antagonists' cause was unlikelihood. The impairment in animal growth rate as a result of antagonists addition has been reported to be accompanied by decrease in feed intake (Humphries *et al*., 1983; Phillippo *et al*., 1987a), which was not affected in the current study.

In the current study, final liver Cu concentration averaged between 192.3 and 127.1 mg/kg DM in lambs fed diets supplemented without or with antagonists and these concentrations are well in excess of the 20 mg/kg DM considered to be marginal for Cu deficiency (Suttle, 2010). Liver Cu status was different between lambs fed a diet, with a high liver Cu concentration in urea WCW and GS compared with fermented WCW fed lambs. The effect of forage type on liver Cu status has been also reported in dairy cows (Sinclair *et al*., 2017) who reported that liver Cu concentration in cows fed maize silage was higher than those fed grass silage. The higher liver Cu concentration in GS compared to fermented WCW may be due to the higher dietary Cu intake in GS compared with fermented WCW (Table 6.4), as the increase in Cu intake can result in increased liver Cu concentration (Suttle, 2012). Cu intake in urea WCW fed lambs was similar to fermented WCW fed lambs and lower than GS, but compared with GS, liver Cu status in lambs fed urea WCW was numerically higher (approximately 17 mg/kg DM) and significantly higher than fermented WCW. The application of urea has been found to raise the DM digestibility (Ørskov *et al*., 1983). Adesogan *et al*. (1998) reported that the digestibility of organic matter content in DM of urea WCW harvested at DM of 620 g/kg was (646 g/kg), which was higher than that of fermented WCW (611 g/kg) harvested at lower DM 593 g/kg. Likewise, Walsh *et al*. (2008) also reported DM digestibility of urea WCW was higher compared with fermented WCW and GS in dairy cows. In addition, Cu in feedstuffs has been reported to be mainly associated with the lignocellulose (Ibrahim *et al*., 1990) and alkali treatment can dissolve the lignocellulosoic cross-linking of the cell (Hill and leaver, 1999), therefore, may allow for a greater Cu available for absorption by animals. Similarly, ammonia application has been found to decrease the NDF fraction, due to the partial solubilisation of hemicellulose (Haddad *et al*., 1994). This once again, could potentially allow for greater Cu availability.

Consequently, better digestibility in urea WCW may result in increased Cu available for absorption. However, the digestibility of forages in the current study was not determined.

The adverse effect of Cu antagonists on liver Cu status is well documented (Gooneratne *et al*., 1989a; Suttle, 2010). In the current study the inclusion of Mo and S dramatically reduced liver Cu status and this is in agreement with the results of other Chapters in this thesis. This reduction in liver Cu status may be due to the formation of insoluble Cu-thiomolybdate complexes (Dick *et al*., 1975), which is biologically unavailable to the tissues, and reduces Cu availability by reducing Cu absorption and increased biliary Cu excretion (Robinson *et al*., 1987; Crosby *et al*., 2004). As a consequence, liver Cu concentration is depleted (Gooneratne *et al*., 1994; Suttle and Small, 1993; Gooneratne, 2012). Alternatively, liver Cu status also may be decreased systemically due to absorbed thiomolybdates into the blood stream, resulting in sequestering Cu from hepatocytes (Gooneratne, 2012), or rendering Cp to be recycled back to liver, hence reducing liver Cu (Williams, 2004). However, systemic effects of absorbed thiomlybdate on liver Cu status has been found to be evident by an increase in Pl-Cu concentration and reduction in Cp activity, which were not affected in the current study. Therefore, the reduction in liver Cu status in the present study was probably attributed to the formation of the rumen Cu-thiomolybdate complex that may cause a reduction in Cu availability (Suttle, 1991).

The effect of forage type on the degree of thiomolybdate formation had been reported to be affected by the forage type and preservation methods, though, the reason for this effect is not fully understood (Suttle, 1983a; Ivan, 1993; Suttle, 2010). For example, the inhibitory effect of Mo on grass hay was less pronounced compared with fresh herbage or semi-purified diet for lambs, possibily due to the lower release of Cu from hay into the rumen, the site of Cu antagonisms (Suttle, 1983b). Similarly, Sinclair *et al*., (2017) showed that addition of Mo and S resulted in a greater reduction in liver Cu retention in cows fed grass silage compared with maize silage, but the reason for this effect was not clear. In the current study, the antagonist effect of the addition of Mo and S was greater in urea WCW and GS fed lambs compared with fermented WCW fed lambs. The low response to Cu antagonists in fermented WCW compared with the urea WCW may be related to a higher Cu release form urea WCW due to its higher digestibility as discussed above and hence more Cu available in the rumen to complex with Mo and S, thereby reducing Cu availability (Suttle, 1983b). Compared with the grass silage, may be related to the difference in rumen pH between two the forages, which in turn, may affect the interaction between Cu and thiomolybdates (Suttle, 1991) as rumen pH in GS tended to be higher than fermented WCW and urea WCW.

In general, rumen pH can be decreased by feeding more rapidly digested starch such as wheat (Grant, 1994). In addition, the higher NDF content in GS forage compared with other forages (Table 6.5), may increase the residence time of GS in the rumen and increase salivation, resulting in a less acidic rumen environment (Ørskov, 1987). Crosby *et al*. (2004) demonstrated that compared with lambs fed diet unsupplemented with Mo, addition of Mo (4mg/kg DM) resulted in decreased liver Cu concentration to a greater extent (52%) in lambs housed in straw bedded floor compared with lambed housed in expanded metal floor (21%), when they attributed to a higher rumen pH caused by feeding straw. The higher rumen pH should promote a healthy rumen micro-organisms, including protozoa (Ørskov, 1987; Belanche *et al*., 2016), reduces S to sulfide (Suttle, 1995; Spears, 2003; Drewnoski *et al*., 2014). Independent from its essential role in the mechanism by which Mo reduces Cu availability (Dick *et al*., 1975), sulfide also can reduce Cu availability via formation of insoluble Cu-sulfide complexes in the gut (Suttle, 1974; Ivan *et al*., 1985). Moreover, Suttle (1991) suggested that the antagonist effect of thiomolybdate on liver Cu could be reduced by a decrease in rumen pH due to the increase in sulfide absorption (Bray *et al*., 1975) and breakdown thiomolybdates.

Molybdenum in the diet is absorbed as water-soluble molybdates (Suttle, 2010), which are normally stored in tissues such as liver, kidney, and the adrenal gland. Molybdoprotein binds to sulphite oxidase in the mitochondrial membrane, and to dehydrogenase and aldehyde oxidase in the cytosol (Johnson, 1997). Liver Mo concentration was higher in lambs fed WCW diets compared with GS. This possibly arises from a higher DMI by lambs on WCW treatments as Mo concentrations in the forages were similar. Pervious work has reported that when Mo is given as Mo and S or tetra-thiomolybdate, or as ammonium molybdate there is a resultant increase in Mo retention (Suttle and Field, 1983; Zhou *et al*., 2011). However, Sinclair *et al*. (2013) reported a small effect of additional Mo and S in dairy cows on liver Mo concentration. In the current study, additional Mo and S increased liver Mo retention dramatically, which suggests that liver is possibly a major repository for Mo in growing lambs.

In the current study, liver Fe status was greater for lambs fed either of the silages (GS and FWCW) than urea WCW diets. The same effect was observed in Pl-Fe concentration. By the end of the study, only lambs on urea WCW diet lost liver Fe concentration. Ibrahim *et al*. (1990) demonstrated that the acidic environment of ensiling grass silage and maize silage promoted elevated the solubility of Fe and other minerals compared to unfermented feed. Similarly, Hansen and Spears (2009) showed that inclusion of soil to the green chop

before ensiling, resulted in an increased Fe solubility or bioaccessibility compared with the addition of soil after ensiling. Therefore, by ensiling forages GS and FWCW, Fe availability may be increased and have resulted in increased liver and plasma Fe status in the lambs fed ensiled forages. However, the Fe concentration in the present study unlikely to have had an antagonist effect on Cu metabolism (Williams, 2004; Sefdeen *et al*., 2014; 2016). Liver status of Mn was not affected by dietary treatment. Additionally, only a small effect of additional Mo and S on liver Zn concentration was observed in the present study. In general, it has been suggested that the liver is not a major store for these elements (Suttle, 2010; Sinclair *et al*., 2013).

In the current study the mean Pl-Cu concentration (16.5 µmol/L) and Cp activity (17.1 mg/dL) were in excess of the values of 9.4 µmol/L for Pl-Cu concentration and 15 mg/dL for Cp activity that are considered to be deficient in lambs (Kendall *et al*., 2000; Suttle, 2010). Additional Mo and S had no significant effect on Pl-Cu concentration, and only had a small effect on Cp activity which was reduced on one time point. Therefore, the results of the current study confirm the insensitivity of Pl-Cu concentration as an indicator to assess Cu deficiency in ruminants (Ivan, 1993). This is due to maintaining Cu level in plasma within normal range, during depletion or repletion by changing liver Cu concentration (Laven and Livesey, 2005). In growing cattle, a meta-analysis, on the relationship between dietary Cu, Mo, and S and Pl-Cu concentration, Dias *et al*. (2013) concluded that any prediction equation would be limited, Pl-Cu concentration should only be used as an indicator of Cu status when liver Cu stores are either very high or low (Laven and Livesey, 2005; Suttle, 2010). Alimon *et al*. (2011) reported that supplementation of Mo and S had no effect on sheep Pl-Cu concentration. Likewise, Suttle (2012) reported no effect of additional 2 mg/kg DM of Mo and 3 g/kg DM of S on Pl-Cu in Texel ram lambs over a period of 96 days. Plasma concentration of Zn and Mn was not significantly affected by dietary treatment, which is in agreement with the results of Alimon *et al*. (2011) and Sinclair *et al*. (2013; 2017) in sheep and cows respectively.

The ratio of Cp:Pl-Cu was not affected by dietary treatment, except of two time points where it was reduced by the addition of antagonists. The Cp:Pl-Cu ratio in the current study was generally low. The low ratio of ratio of Cp:Pl-Cu concentration (0.6-1.0) also has been observed in cattle fed a diet containing low Mo (1.44-2.1 mg/kg DM) compared with the concentration used in the current study, which reached approximately 5 mg/kg DM in the supplementary diets. The reduction of Cp:Pl-Cu ratio by addition of Mo has been reported to be accompanied by an increase in Pl-Cu concentration, which was not affected by

antagonists in the current study, as an indication of the presence of thiomolybdate in the blood (Williams, 2004). The Cu-containing enzyme (SOD) activity, which is involved in the defence against free radicals in the body (Suttle, 2010) also was not affected by dietary treatment.

6.5. Conclusion

The results of this study indicate that addition of Mo and S had no effect on lambs performance, but lambs fed urea WCW were heavier compared with lambs fed grass silage or fermented WCW. Liver Cu status was numerically higher in lambs fed urea WCW compared with lambs fed GS despite a higher Cu intake in the GS fed lambs. In addition, Cu intake was similar in both urea WCW and fermented WCW fed lambs, but liver Cu status was higher in lambs fed urea WCW than fermented WCW. The higher liver Cu in urea WCW lambs may possibly be attributed to an increase in the WCW digestibility as a result of urea application and hence increase the availability of Cu for absorption due the break down of plant cell wall releasing Cu. The addition of Mo and S had no effect on liver Cu retention of lambs fed fermented WCW, whilst substantially reduced liver Cu retention in lambs fed urea WCW or GS. Liver and plasma Fe status was higher in lambs offered silages (GS and fermented WCW). This confirms the findings that the bioavailability of Fe increased after ensiling forages (Hansen and Spears, 2009). A small effect was observed of adding of antagonists, within limits of this study, on plasma Cu or indicators of plasma Cu activity, and it can be concluded that using these parameters to assess Cu status is limited.

To conclude, the use of urea to preserve WCW would be more beneficial to growing lambs in terms of increasing in weight gain and Cu availability, however, during the presence of high levels of dietary Mo and S it may be less beneficial than preserving WCW as fermented WCW. The reason for the difference in the Cu metabolism between lambs fed FWCW and lambs fed GS and urea WCW may be related to the rumen pH and its influence of Cu and thiomolybdate interactions. However, more research is required to elucidate the role of rumen pH in Cu metabolism in ruminant animals.

Chapter 7 The effect of grass preservation method on Cu distribution in rumen fluid following *in vitro* fermentation

7.1. Introduction

Results obtained from previous Chapters (3, 4, and 6) have demonstrated the effect of forage type on liver Cu status but the mechanism of this effect remains unclear. As discussed previously, the difference in liver Cu status may either be attributed to the difference in Cu intake, the effect of rumen pH on Cu x antagonists interactions, or the difference in Cu distribution between rumen digesta fractions.

The coefficient of Cu absorption has been found to vary between feedstuffs and the preservation of grass as hay or silage generally improves Cu availability (Suttle, 1986). Fisher *et al*. (1972) was the first to evaluate the effect of forage preservation on plasma Cu concentration in cattle, with concentrations being higher when offered hay compared with those offered grass silage. Similarly, Suttle (1980a; 1980b; 1983b) reported that Cu availability based on plasma Cu concentration in lambs and ewes were higher when hay or dried grass was offered compared to grass silage or fresh grass. However, the reasons for the effects of preservation method on the Cu availability was not clear, but may possibily be was due to the lower extent to which Cu was released from plants into the rumen, which is the site of Cu interactions with its antagonists (Suttle, 1983b). The release of minerals was found to be different between four different species of tropical hays digested in nylon bags in the rumen and the proportion of minerals removed during digestion were positively related to the initial mineral in the hays (Playne *et al*., 1978). Waghorn *et al*. (1990) demonstrated that the proportion of Cu in the supernatant fraction of the rumen digesta was generally lower with dried feed than fresh feed. In contrast, in Chapter 5 show the higher Cu distribution in supernatant fraction in grass and maize silag compared with dried grass pellets. Waghorn *et al*. (1990) concluded that the difference in minerals distribution appears to be dependent on concentration of minerals in the feed DM rather than type of feed. Therefore, the aims of this study were to investigate the effects of grass preservation method on Cu distribution in rumen liquor following *in vitro* fermentation and to investigate whether the different in Cu distribution of the rumen digesta fractions due to the different in Cu content between forages or due to the difference in Cu release from plant materials.

7.2. Materials and methods

7.2.1. Experimental design, forage production and chemical composition

The experiment was conducted on grass forage preserved differently as hay, silage, or fresh grass. The experiment was designed as a one-way ANOVA. Forage was obtained as a first cut grass from a predominately perennial ryegrass sward. Grass was harvested on 25th May 2016, using a self-propelled, precision-chop forage harvester (John Deer, 7480I, UK), and wilted for 24 hrs, and chopped at 10 mm. Approximately 4 kg of the chopped grass was frozen and kept at –20°C prior to being used as a control or fresh grass (FG), and another 4 kg was dried in an air forced oven at 25°C for 5 days to produce artificial grass hay (AGH), while grass silage (GS) was produced in a small silos. Four silos were lined with a plastic bag and filled to the neck with approximately 2 kg of forage. The neck of the plastic bag was sealed with tape, and approximately 1 kg of sand was placed on the top of each silo in order to consolidate well. Silos were ensiled for four weeks before being opened, and stored at -20°C before being used. Forages (FG, GH, and GS) samples were analysed for dietary content of DM, Ash, CP, NDF, EE, and pH as described in Chapter 2, sections; 2.1.1 to 2.1.6. Dietary mineral content of the experimental forages were also determined as described in section 2.4.1. Chemical composition and minerals of the experimental diets are presented in Table 7.1.

Table 7.1. The effects of preservation methods on the chemical composition of fresh grass (FG), grass silage (GS), and artificial grass hay (AGH).

Item	FG	GS	AGH
Chemical composition, g/kg DM			
DM, g/kg	245	216	920
CP,	91	90	91
NDF,	491	524	561
EE,	9.5	13.1	13.4
Ash,	79	97	81
NSC[2],	329.5	275.9	253.6
pH[3],	nd	3.99	nd
Mineral concentrations, mg/kg DM			
Cu,	6.2	6.3	6.5
Mo,	0.6	0.7	0.8
S, g/kg DM	0.6	0.8	0.6

[1] nd= not detected.

[2] Non-structural carbohydrate (NSC) was calculated by subtracting the sum of the amounts (g/kg) of CP, NDF, EE, and ash from 1000 (McDonald *et al*., 2011)

7.2.2. *In vitro*

The distribution of Cu and other minerals in the rumen digesta were determined by using two *in vitro* methods, including the gas production methods to mimic the rumen (Theodorou *et al*., 1994), and the two-stage method to mimic the abomasum (Tilly and Terry, 1963).

7.2.2.1. *In vitro* gas production

Forages (FG, GS, and AGH) were fermented using an *in vitro* gas production batch culture technique as described in Chapter 5 section (5.2.2). All three forages were incubated (at 19th of June 2016) in triplicate and replicated in four separate weeks. Three blank vessels were also incubated in each run in order to correct gas production. The experimental design had 9 fermentation vessels with 3 vessels for each forage.

7.2.2.2. Two-stage method

The *in vitro* two stage incubation method was adapted from Tilly and Terry (1963) was used. In the first stage, the same method of *in vitro* gas production was used as described in section 7.2.2.1. The second stage involved digestion with pepsin-HCl for 48hrs at 38°C (Tilly and Terry, 1963). After 48hrs of incubation vessels were removed from the incubator and dried in an air force oven at 60°C for 5 days. Vessels were then filled with 200 ml of freshly made pepsin-HCl solution, which was prepared by dissolving 2 g of pepsin (1:10,000 biotechnology grade; Lutterworth, Leicestershire, UK) in 850 ml of purite water, and added to 100 ml of 1M HCl, and the solution was made up to 1L with purite water. Vessels were then incubated at 48°C for 48hrs, and were occasionally shaken by hand. The experimental design had 9 fermentation vessels with 3 vessels for each forage.

7.2.3. Vessel pH determination

At the end of each run (after 48hrs), the lids were removed from all vessels and the pH was directly determined, as described in a section 2.1.6.

7.2.4. Fractionation of vessels fluid

Fermented rumen liquor was fractionated into 4 fractions, including a strained solid fraction, protozoa rich fraction, bacteria rich fraction, and supernatant fraction as described in section 5.2.4. Similarly, at the end of second 48hrs of pepsin-HCl digestion, vessel contents were

centrifuged at 30,000 g for 30 min and the supernatant was collected for subsequent mineral distribution determination in the strained fraction (SN).

7.2.5. Mineral analysis of vessel fractions

Samples from all collected fractions were prepared for mineral analysis as described in section 5.2.5. Following that samples were analysed for mineral concentrations as described in section 2.4.1. Samples of the SN fraction in both gas production and pepsin digested vessels were directly analysed for mineral content, as described in section 2.4.1. The mixture of rumen fluid:saliva was not analysed for its meniral content.

7.2.6. Statistical analysis

Cumulative gas production was analysed using repeated-measures as one way design (ANOVA) with the treatment being forage type. Vessel pH and mineral distribution were also as a one-way ANOVA design with the treatment being forage type. Runs were used as a block. Data for this study was analysed using Genstat 17th edition (Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Significance was set at $P<0.05$ and trends at $P<0.10$. Significant differences between means were tested using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

7.3. Results

7.3.1. Gas production

Repeated measures analysis indicated that there was an effect of time on cumulative gas production (Fig. 7.1). Gas production increased (P<0.001) in all forage treatment by the end of the experiment. However, there was no time x forage interaction on cumulative gas production (P>0.05).

There was a difference (P<0.001) between forages in cumulative gas production, with gas production being higher in fresh grass and grass silage compared with artificial grass hay from time 3h until 48hrs.

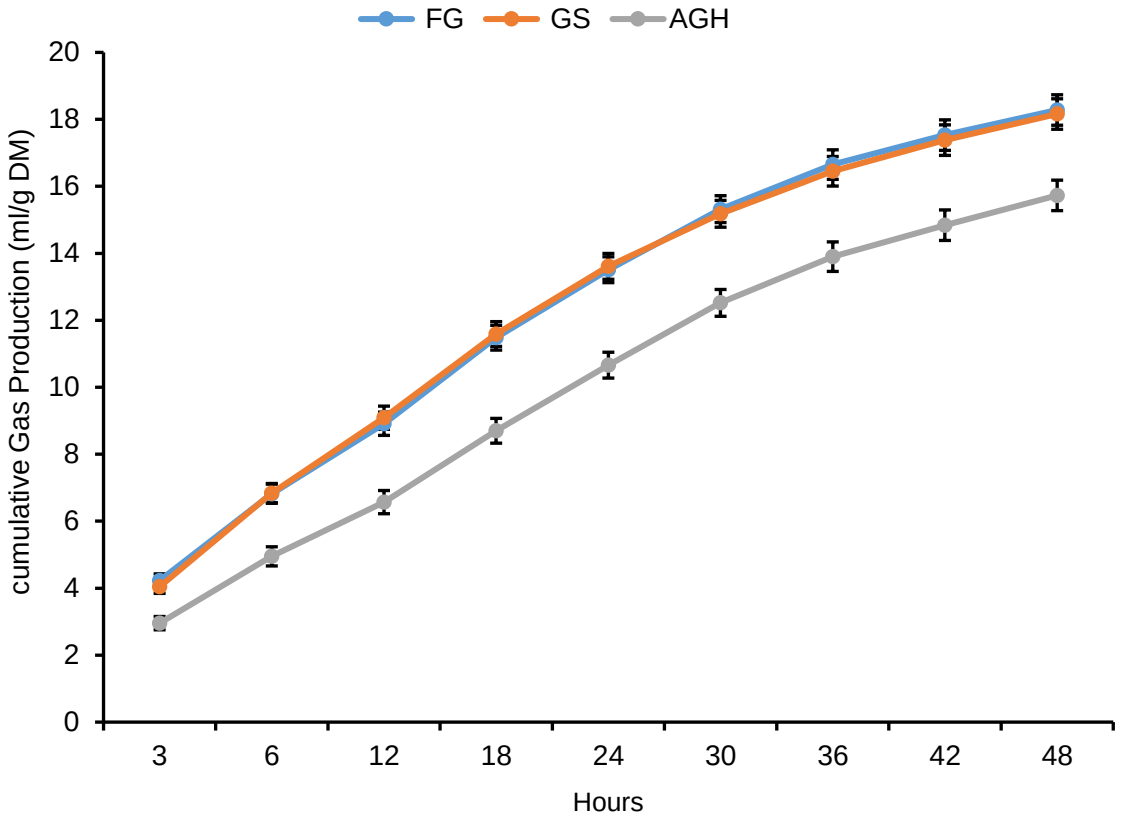


Figure 7.1. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the in vitro cumulative gas production over a period of 48hrs at 39°C. Error bars indicate SED. Individual weekly data have been analysed by ANOVA, but caution should be exercised when interpreting individual means when the time x forage interaction is not significant.

Repeated measures:	s.e.d	P-value
Time effect	0.524	<.001
Time x Forage effect	0.92	0.916

7.3.2. Vessel pH

The final pH by the end of the study was affected (*P*<0.001) by preservation methods (Fig. 7.2). The grass silage and fresh grass forages had a lower pH compared with artificial grass hay, with mean values of 5.96, 5.98, and 6.08 (s.e.d, 0.021) respectively.

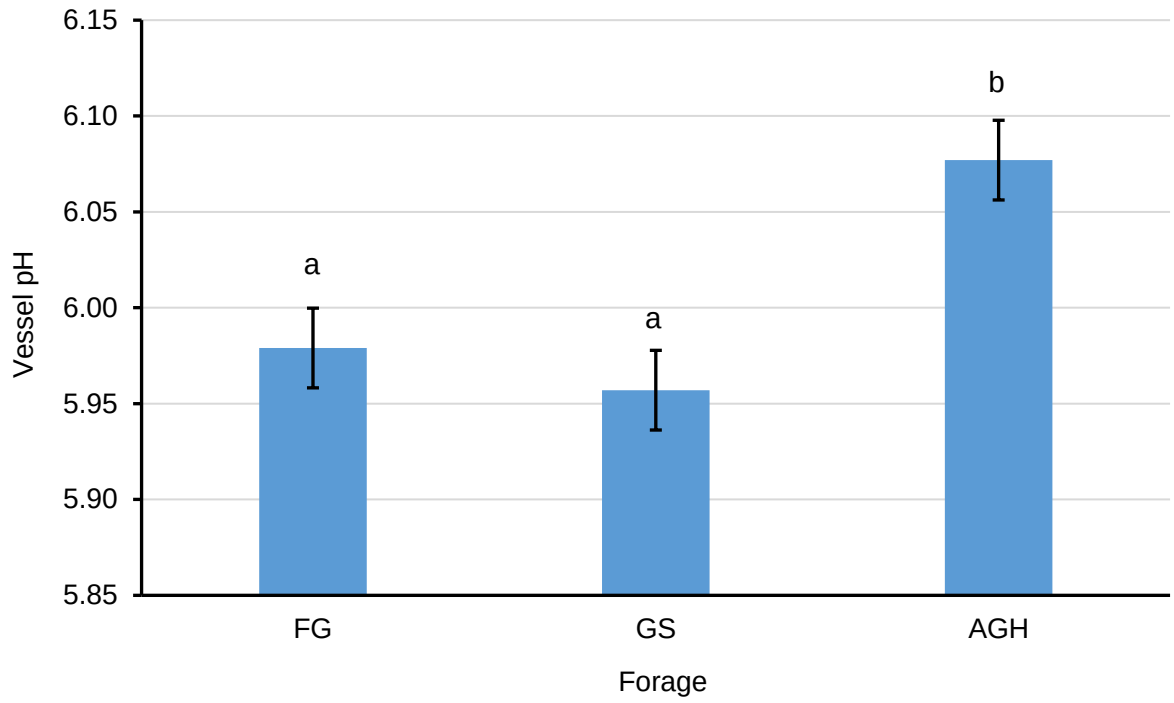


Figure 7.2. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the final vessel pH over the period of 48hrs at 39°C. Error bars indicate SED. [a,b] Means with different superscripts are significantly different (*P*<0.05).

7.3.3. Distribution of minerals within fluid fractions

7.3.3.1. Distribution of copper within fluid fractions

Cu distribution in the strained solid, protozoa rich, and bacteria rich, and supernatant fractions was not affected by preservation method, and there was no (P>0.05) difference between fresh grass, grass silage, and artificial grass hay in the associated Cu with strained solid, protozoa rich, and bacteria rich, and supernatant fractions (Table 7.2).

Table 7.2. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the percentage distribution of copper in fractions of *in vitro* gas production fermented rumen digesta (%)[1].

Fractions	Forages			Significance[2]	
	FG	GS	AGH	s.e.d	P value
SS	37.4	39.9	38.5	2.56	0.634
PR	27.6	26.7	28.2	1.57	0.622
BR	26.3	23.9	24.2	1.89	0.392
SN	8.6	9.5	9.1	1.11	0.741

[1] SS= strained solid fraction; PR= protozoa rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[2] s.e.d= standard error of difference.

7.3.3.2. Distribution of molybdenum within fluid fractions

In the supernatant fraction, the proportion of Mo was higher (P<0.05) in artificial grass hay forage compared with fresh grass or grass silage (Table 7.3). The distribution of Mo in strained solid, protozoa rich, and bacteria rich fractions was not different between forages (P>0.05).

Table 7.3. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the percentage distribution of molybdenum in fractions of *in vitro* gas production fermented rumen digesta (%)[1].

Fractions	Forages			Significance[2]	
	FG	GS	AGH	s.e.d	P value
SS	33.7	34.5	29.5	2.59	0.132
PR	27.2	27.0	28.0	1.45	0.804
BR	29.2	28.6	29.6	2.19	0.916
SN	9.8[a]	9.8[a]	13.0[b]	0.79	<0.001

[1] SS= strained solid fraction; PR= protozoa rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[2] s.e.d= standard error of difference.

7.3.3.3. Distribution of sulfur within fluid fractions

The proportion of S in the supernatant fraction of artificial grass hay tended to be higher compared with fresh grass or grass silage (*P*=0.080) (Table 7.4). However, the proportion of S in the strained solid, protozoa rich, and bacteria rich fractions was not different (*P*>0.05) between forages.

Table 7.4. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the percentage distribution of sulfur in fractions of *in vitro* gas production fermented rumen digesta (%)[1].

Fractions	Forages			Significance[2]	
	FG	GS	AGH	s.e.d	P value
SS	28.2	30.1	27.9	2.30	0.588
PR	26.3	24.9	27.0	1.49	0.364
BR	35.8	35.5	34.1	2.19	0.694
SN	9.6	9.5	11.1	0.74	0.080

[1] SS= strained solid fraction; PR= protozoa rich fraction, BR= bacterial rich fraction, SN= supernatant fraction.

[2] s.e.d= standard error of difference.

7.3.4. Distribution of minerals released to supernatant fraction in two-stage fermentation

The percentage of Cu released to supernatant fraction in vessel fluid after being digested with pepsin-HCl were not different (P>0.05) between fresh grass, grass silage, and artificial grass hay (Table 7.5). However, Mo released to the supernatant fraction was higher (P<0.001) in artificial grass hay compared with fresh grass and grass silage. Similarly, release of S to supernatant fraction was affected (P<0.05) by forage type, with a higher proportion in artificial grass hay, intermediate in grass silage and lowest proportion in fresh grass.

Table 7.5. The effect of forage type, fresh grass (FG), grass silage (GS), and artificial grass hay (AGH) on the percentage distribution of mineral in the supernatant fraction of *in vitro* two- stage fermentation of rumen digesta.

Minerals, %	Forages			Significance[1]	
	FG	GS	AGH	s.e.d	P-value
Cu	12.4	13.8	13.7	2.54	0.482
Mo	11.5[a]	12.1[a]	17.0[b]	1.23	<0.001
S	29.4[a]	30.0[ab]	32.6[b]	1.28	0.045

[1] s.e.d= standard error of difference.

[a,b] Means within a row with different superscripts are significantly different (P<0.05).

7.4. Discussion

In the current study, the NDF content was highest in the AGH and lowest in FG, while GS was intermediate. The higher NDF content in AGH compared to others is in agreement with the results of Salamone *et al*. (2012) and Belanche *et al*. (2016). It is suggested that the incorporation of soluble carbohydrates into the NDF through the Maillard reaction may be attributed to the high NDF content in grass hay (Salamone *et al*., 2012). The lower NDF content in GS is possibly due to hydrolysis of soluble carbohydrates and hemi-cellulose during ensiling. Similar results were reported by Salamone *et al*. (2012). Moreover, degradability of protein has been reported to be greater in grass silage compared to hay or wilted grass due to the extensive protein break down during the ensiling process (Petit and Tremblay, 1992). The source of gas during *in vitro* incubated feedstuffs with buffered rumen fluid is mainly comes from the fermentation of carbohydrates, (mainly CO_2 and CH_4) (Getachew *et al*., 1998). Therefore, feedstuffs with high carbohydrates and protein degradability may produce higher amount of gasses. Similarly, gas production results in the present study for FG and GS were higher compared with AGH, which is in accordance with the results of Huntington *et al*. (1998), Brown *et al*. (2002) and Gosselink *et al*. (2004) who reported that GS and FG had a higher gas production compared with hay. Brown *et al*. (2002) suggested that the high level of structural carbohydrates in the hay may have resulted in slower fermentation and lower gas production.

The higher gas production in FG and GS compared with AGH in the present study showed promoted a more acidic fermentation characterised by a higher reduction in vessel pH in comparison with GH, which is in line with findings reported by Benlache *et al*. (2016) who showed that FG forage had a lower pH compared with grass hay when incubated *in vitro* for 48hrs. Benlache *et al*. (2016) suggested that lower vessel pH in fresh grass possibly related to the greater availability of easily fermented carbohydrates with fresh grass compared with grass hay. In the current study, the content of NSC was lower in AGH compared with FG or GS. Therefore, the higher gas production and lower pH observed between FG and GS compared with and AGH may be related to the higher availability of easily fermented carbohydrates in FG and GS (NSC) and lower content of structural carbohydrates in comparison with AGH.

The results of the current study demonstrated that Cu, Mo, and S were mainly (approximately 90%), associated with the solid phase (strained solid, bacteria rich, and protozoa rich fraction) at the expense of the supernatant fraction (liquid phase). These results are consistent with the results of Chapter 5, where Cu, Mo, and S were

predominately associated with the solid phase. Similarly, the predominant association of Cu and antagonists with the solid phase has been reported by several authors in studies conducted *in vitro* or *in vivo* (Grace and Suttle 1979; Allen and Gawthorne, 1987; Waghorn *et al*., 1990). The reduction in Cu availability within the digestive tract has been associated with a number of factors among them is the association of Cu with the solid phase of the rumen digesta. However, there was no suggestion of a clear mechanisms by which these operate (Bremner, 1970; Allen and Gawthorne, 1987; Waghorn *et al*., 1990). Price and Chester (1985) demonstrated that the relative Cu availability in the dried grass fed to sheep was 75%, and Cu availability of the rumen digesta, collected from sheep fed the same diet and given to rats, was substantially reduced up to 12%. Price and Chester (1985) concluded that factors limiting Cu availability mainly associated with the solid phase of the digesta, where Cu form insoluble complex by associating or incorporating into the bacteria, protozoa, or undigested plant material (solid phase) that Cu may be not released even under acidic condition as found in the abomasum (Waghon *et al*., 1990). In the current study, distribution of Cu in rumen digesta fractions was not affected by preservation methods and this is consistent with the findings of Waghorn *et al*. (1990) who demonstrated that the distribution of Cu in the solid phase was not different between six different forages (three fresh and three dried).

The disassociation of Cu from the solid phase is important for absorption, as minerals require to be in a soluble form for absorption (Bremner, 1970). The release of minerals from the solid phase in the abomasum that has lower pH than rumen has been in part attributed to the rupture of micro-organisms and release of their mineral content into the abomasum (Waghorn *et al*., 1990). In the current study, pepsin-HCl digestion only slightly increased distribution of Cu in the supernatant fraction and forage type had no effect on Cu distribution which is in accordance with the results of Waghorn *et al*. (1990). Waghorn *et al*. (1990) concluded that the difference in Cu distribution in rumen and abomasum digesta was mainly related to the difference in Cu concentration in feed DM rather than difference between forages in releasing Cu from plant materials. In the current study, the Cu content were similar between FG, GS, and AGH. Therefore, no effect of preservation method of grass on Cu distribution either in fermented rumen or pepsin-HCl digested rumen digesta, seems to suggest that the concentration of Cu in feed DM may have a major effect on the Cu distribution and availability of Cu for absorption by animals. In contrast, the distribution of S was substantially increased after pepsin-HCl digestion from approximately 9% to 30%, which may contribute to reduce Cu absorption. Sulfur reduced to sulfide in the rumen is belived to interact with Cu to form insoluble Cu-sulfide a form of Cu that is unavailable to ruminants. In sheep, increasing dietary S in the organic or inorganic form reduced Cu bioavailability by 30-56%.

In the current study, the higher distribution of Mo in SN fraction, of AGH compared with FH and GS, was observed in both fermented rumen liquor and pepsin-HCl digested. In Chapter 5, Mo distribution in SN the fraction was also higher in grass haylage compared with dried grass pellets, grass and maize silages. The reason for this effect was not clear, but may be due to the difference in mineral release from plants. Playne *et al*. (1978) reported that the difference in the rate of release minerals from 4 tropical hay during their digestion in nylon bag in the rumen of cattle. It has been shown that grass hay tended to have a greater NDF and ADF disappearances compared with fresh grass (Belanche *et al*., 2016) possibly due to the higher concentration of protozoa such as sub family *Entodiniinae* with a high fibrolytic activity (Dehority, 2003), and hence may result in release more Mo into the SN fraction, as the majority amount of minerals are present in the cell wall of plant materials (Ibrahim *et al*., 1990). Moreover, thiomolybdates in the rumen digesta if not bound to the solid phase may be hydrolysed when remaining in the liquid phase (Gould and Kendall, 2011).

7.5. Conclusion

Results from this study indicated that over 88% of Cu and other minerals were found to be associated with the solid phase (strained solid, protozoa rich, and bacteria rich fractions) at the expense of supernatant fraction (liquid phase). This is confirming the involvement of the solid phase of the digesta in reducing available Cu for absorption by ruminants. Preservation fresh grass as hay or silage had no significant effect of Cu distribution in the fermented rumen liquor or after pepsin-HCl digestion, as Cu concentrations between forage were similar. Therefore, the results of the present study support the findings suggest that the differences in distribution of Cu between digesta fractions are potentially related to the difference in the concentration of Cu in the feed DM rather than preservation method.

Chapter 8 General discussion

8.1. Introduction

Copper metabolism in ruminants has been found to be affected by the forage type being offered, with Fisher *et al*. (1972) reporting that feeding hay to cows increased plasma Cu levels compared to grass silage. In ewes, at low dietary Mo concentration (<2 mg/kg DM), the absorption of Cu was higher in hay compared to fresh grass (Suttle, 1983a). In general, the coefficient of Cu absorption has been found to be higher in feedstuffs low in fibre such as cereals and legumes compared with fresh herbage (Suttle, 1983a; 1983b). Also, conservation of forages as hay or silage could improve Cu availability (Suttle, 1986). It is also recognised that the antagonist effect of Mo and S on Cu availability is also changed by basal diet (Suttle, 1980a; 1980b). Early investigations by Ferguson *et al*. (1943) reported that the ‘’teart’’ condition in Somerset pasture was attributed to high level of Mo in the pasture (40-50 mg/kg DM) and it was alleviated when herbage was made into hay. Suttle (1983b) reported that the inhibitory effect of additional Mo on Cu availability in sheep was proportionally less in hay compared to fresh herbage (Suttle, 1983a;1983b;2010). A recent study in dairy cows demonstrated that the reduction in liver Cu retention caused by addition of Mo and S was greater in grass silage than in maize silage based diets (Sinclair *et al*., 2017). The reasons for these differences were not clear, but it could be attributed to the effect of rumen pH and its influence on thiomolybdate formation (Gould and Kendall, 2011). Suttle (1991) discussed that increasing dietary Mo did not accelerate the rate of liver Cu depletion in sheep fed a whole grain diet possibly due to the lowering rumen pH by feeding a high fermentable carbohydrate diet that lowered rumen pH, resulting in increased absorption of sulfide and break down of thiomolybdates. Allen and Gawthorne (1987) reported that thiomolybdates bind with the solid phase of rumen digesta that allows them to avoid hydrolysis from the acid solution of the abomasum. Therefore, three lamb studies, and two *in vitro* studies were undertaken within this thesis that were designed to further investigate the antagonist effects of Mo and S fed with different forages on Cu metabolism in lambs.

These series of studies were designed to examine the effects of forage type and Mo and S on Cu metabolism in sheep. The results from studies 1 and 2 clearly demonstrated that liver Cu status was affected by different forages fed to lambs and the higher liver Cu status was in lambs that had a lower rumen pH. Addition of Mo and S substantially reduced liver Cu status (study 1 and 2) and plasma Cu status (study 2). In both studies 1 and 2 no interaction between forage type and Cu antagonists were observed. *In vitro* work (study 3) was then conducted using the same forages that were used in the previous studies to investigate

whether the reduction in Cu metabolism in (study 1 and 2) was due CuxMoxS interactions in the rumen reducing available Cu for absorption, and depleting liver Cu or reducing plasma Cu concentration. It was found that Cu and its antagonists were mainly associated with the solid phase and the addition of Mo and S markedly reduced Cu in supernatant fraction by incorporation or binding Cu into the solid phase. It was concluded that the adverse effect of antagonists on Cu metabolism in Chapters 3 and 4 may be related to the formation of insoluble Cu-thiomolybdate complexes, and excreted via faeces. Another study was conducted (study 4) to investigate the effect of preservation of whole crop wheat (WCW) as fermented or urea-treated and grass silage on rumen pH and their interaction with the addition of Mo and S. Results from study 4 showed that rumen pH in grass silage fed lambs tended to be higher compared with other forages, and urea WCW and grass silage fed lambs had a higher liver Cu status compared with those fed fermented WCW. However, liver Cu retention in fermented WCW fed lambs was not affected by addition of antagonists, whereas, in both urea WCW and grass silage fed lambs it was significantly reduced. It was concluded that the lack of effect of antagonists on liver Cu retention in fermented WCW may possibly be attributed to the effect of the low rumen pH in fermented WCW fed lambs contributed to the reduction in the production of thiomolybdates. The final study (*in vitro*), aimed to investigate the difference in Cu release from forages whether related to the different in Cu release from forages or Cu concentration in DM of the feed. No difference in Cu distribution was observed between fresh grass preserved as hay or silage, or release of Cu from solids in to the supernatant after pepsin-HCl digestion of the rumen digesta.

8.2. Animal performance characteristics

Lamb performance in this thesis was examined to evaluate the effect of forage type and antagonists. However, the main aim of this thesis was to study Cu metabolism and its influence by forage type and antagonists. The maize silage forage fed lambs compared with dried grass pellets (Chapter 3) or grass haylage (Chapter 4) showed a higher liveweight gain than lambs fed the other forages. Ware and Zinn (2005) and Salinas-Chavira *et al.* (2013) observed a lower in weight gain of steers fed pelleted diets compared with ground straw. The lower FCE in dried grass pellets fed lambs (Chapter 3) compared with maize silage reflects the poorer utilisation of energy with dried grass pellets than maize silage (Salinas-Chavira *et al.*, 2013). Pelleting diets has been found to reduce the utilisation of energy and feed digestibility in animals (Boucque *et al.,* 1973; Thomson and Beever, 1979; Knaus *et al.*, 1999), possibly due to an increase in the proportional outflow of particulate matter from the rumen by feeding pelleted diets (Boucque *et al.*, 1973).

Within a given feed, NDF had been reported as a good measure of feed quality and plant maturity. For grass forages, an NDF content above 600 g/kg DM would be considered as low quality (Van Saun, 2006). In grass haylage (Chapter 4) the NDF content was higher than 600 g/kg DM, suggesting a poor quality of grass haylage. In addition, compared with maize silage, the NDF content of grass haylage was greater by 160 g/kg DM. It has been reported that increasing NDF from 40 to 80 g/kg DM in forages such as alfalfa forage fed to cattle has been shown to reduced final liveweight from 605 to 588 kg respectively, gain efficiency from 0.181 to 0.177 (g/kg DMI) and dietary net energy required for gain from 6.82 to 6.53 (MJ/kg) (Salinas-Chavira *et al*., 2013). The basis of the NDF effect on weight gain has been attributed to a reduced utilisation of energy as a result of dilution of dietary energy by increasing NDF and reduced OM digestibly (Salinas-Chavira *et al*., 2013). It was recognised that ME derived from poorly digested forages, such as straw and low quality hay, was utilised for growth with low efficiency between 0.20-0.40 (MJ/kg). This low efficiency was attributed to the ‘work of digestion’, the energy required for mastication of fibrous feeds and propulsion of their undigested residues through the gut (McDonald *et al*., 2011).

Keady (2005) from the 9 comparisons in beef cattle reported that the performance of animals as indicated by daily liveweight gain was significantly increased (+0.23 kg/d) by replaceing grass silage (totally or partially) with maize silage. A few studies have been undertaken to evaluate the effect of maize silage and grass silage or grass haylage on sheep performance (Keady *et al*., 2013). Keady and Hanrahan (2009) showed that ewes fed low feed value maize silage supplemented without or with concentrate at 0.2 kg/d had higher liveweight than of grass silage fed ewes (65.8 and 61.4 kg respectively. The higher

weight gain by feeding maize silage has been attributed to an improved ME utilisation and dry matter digestibility (Keady *et al*., 2006; 2007; Walsh *et al*., 2008).

In recent years, there has been an interest in using whole crop wheat (WCW) silages for feeding animals primarily due to the relative low cost of forage production and benefit in increased DM intake and animals performance (Keady *et al*., 2013). The WCW is an alternative feed for grass, especially in temperate grass grown place that maize silage does not grow well. In Chapter 6, lambs fed urea WCW had higher weight gain and FCE than lambs fed grass silage or fermented WCW. The replacement of grass silage with urea WCW at rate of 75% increased liveweight change from 0.08 kg/d in the grass silage fed cows to 0.36 kg/d (Sinclair *et al*., 2007). Keady *et al*. (2007) concluded that replacing grass silage with fermented WCW resulted in an increased weight gain in beef possibly due to an improved utilisation of ME and increased intake. Likewise, Walsh *et al*. (2008) noted that offering fermented and urea WCW resulted in an increased intake, weight gain and *in vitro* DM digestibility in beef cattle compared with grass silage. Urea or alkaline treatment of WCW resulting in a reduction in NDF fraction, mainly because of solubilisation of hemicellulose (Haddad *et al*., 1995; Hill and leaver, 1999). Therefore, the application of urea may be contributed to an improve weight gain by growing lambs due to a reduced NDF fraction and increased digestibility.

Texel cross store lambs growth rate has been shown to be not affected by the addition of Mo at 10 mg/kg DM and S 2 g/kg DM over a period of 7 weeks in the study Mackenzie *et al*. (2000) who concluded that growth rate may be an insensitive parameter Cu deficiency. Williams (2004) reported no effect of additional Mo (5 and 10 mg/kg DM) on lambs performance over 10 weeks. Alimon *et al*. (2011) and Suttle (2012) did not observe a reduction in lambs' performance during the addition of Mo and S. In Chapter 3, 4, and 6 additions of approximately 5 mg of Mo/kg DM and 4 g of S/kg DM was not expected to reduce lambs performance. In contrast, Humphries *et al*. (1983) and Phillippo *et al*. (1987a; 1987b) reported that additional 5 mg Mo/kg DM reduced growth rate in heifers after 16-20 weeks of feeding. The reason for this effect was not clear, but in both Humphries *et al*. (1983) and Phillippo *et al.* (1987a) studies the reduction in growth rate was accompanied by a decrease in feed intake, and reduction in liver Cu concentration to very low levels (4-5 mg/kg DM). In Chapters 3, 4, and 6 liver Cu concentrations were above 100 mg/kg DM and DM intake was not affected by antagonists. The mechanism by which Mo and S effect feed intake has been proposed to be possibly related to the systemic effects of absorbed thiomolybdates, which in turn, may have a direct effect on Cu-dependent enzymes such as

peptidylglycine α-amidating monooxygenase that exert its influence on the cholecytoinin and gastrin hormones, regulating appetite (Suttle, 2010).

8.3. Composition of the experimental diets

In order to be able to discuss the observed differences due to dietary Mo and S in this series of studies, it is appropriate to compare the diets of the three experiments described in Chapters 3, 4, and 6. The composition of the experimental diets is presented in Table 8.1.

Table 8.1. The composition of copper, molybdenum, and sulfur of the experimental diets (DM)- Chapters 3, 4, and 6 [1].

Chapter 3	Diet			
	DGP-	DGP+	MS-	MS+
Cu, mg/kg DM	9.3	9.5	7.9	7.8
Mo, mg/kg DM	1.90	4.60	2.70	4.80
S, g/kg DM	3.7	4.3	3.5	3.9

Chapter 4	Diet			
	GH-	GH+	MS-	MS+
Cu, mg/kg DM	11	11.6	10.7	10.7
Mo, mg/kg DM	1.80	4.50	1.50	4.20
S, g/kg DM	1.9	3.3	1.8	3.2

Chapter 6	Diet						
	Forage			Concentrate			
	GS	FWCW	UWCW	GS- and UWCW-	GS+ and UWCW+	FWCW-	FWCW+
Cu, mg/kg DM	11.5	6.9	6.7	11.2	11.3	11.3	11.5
Mo, mg/kg DM	1.11	0.88	1.02	1.36	19.54	1.81	19.91
S, g/kg DM	2.7	1.1	1.1	6.0	17.5	7.7	16.4

[1] Mineral composition for the experimental diets for Chapters 3 and 4 was supplying 600 g/kg DM forage and 400 g/kg DM concentrate (60:40). Diets were offered at restricted level at ratio of 60:40 (forage:concentrate).
[2] Feeding system for Chapter 6 was forage (*ad libitum*) and concentrate at 300 g/day. The experimental diets contained approximately 700 g/kg DM forage and 300 g/kg DM concentrate.

The Cu contents of the diets in all three Chapters were similar to the recommendations for sheep as specified in NRC (1985) and NRC (2005). The Cu content in all Chapters also varied between diets. Therefore, this may account for the differences in liver Cu status between forages as described in Chapters 3, 4, and 6 and will be discussed in section 8.4. Molybdenum and sulfur contents of the experimental diets (unsupplemented with antagonists) in Chapters 3, 4, and 6 were approximately 1.5 to 2.5 mg/kg DM of M and 1.5 to 3.5 g/kg DM of S respectively. These levels of Mo and S are reported to reduce Cu availability in the diet as part of a thiomolybdate complex (Suttle, 1974;1983).

The variations in the mineral composition within experimental diets in Chapters 3, 4, and 6 may be attributed to the dietary components used in the formulations of the diets. These differences were due to the availability of feedstuffs and time of year in which the study was undertaken.

8.4. Effect of forage type on Cu status

Dietary composition is important because it determines the proportion of the dietary Cu which is absorbed by animals (its availability) and this proportion can vary widely (Suttle, 1986). Therefore, all studies described within this thesis have been conducted towards the understanding and control of Cu deficiency in ruminants. The availability of Cu has been suggested to be associated with feed type, mineral composition of the feedstuff, the interaction between feed type and mineral composition, and genetic constitution (Suttle, 1983a; 1986; 2010). The effect of forage type on liver Cu status was clearly identified in this thesis. From Table 8.2, it appears that lambs fed forages with a lower fibre content (NDF) and higher easily digested material such as NSC (sugar and starch) had a higher liver Cu status. The impact of a high fibre content in limiting Cu absorption has been related to the irreversible binding of Cu, or possibly due to indirectly increasing the amount of time Cu resides in the rumen environment and the site of CuxMoxS interactions (Suttle, 1983a). In addition, the higher coefficient of Cu absorption in feedstuffs such as cereals (0.10) compared with hay (0.073) or fresh pasture (0.012) (Suttle, 1986) has been attributed to the higher proportion of readily digested carbohydrate (Suttle, 1991).

Table 8.2. Neutral detergent fibre and nonstructural carbohydrate content of the forages used in Chapter 3, 4, and 6.

	DGP	MS	GH	GS	FWCW	UWCW
Chemical composition, g/kg DM						
NDF	426	381	654	588.2	413.6	309.2
NSC	277	475	176	110.1	414	486.7

Using data from Chapters 3, 4, and 6, Fig 8.1 indicates the relationship between the forage NDF content and liver Cu retention for all treatment groups at the end of each respective trial. These results indicate that the regression coefficient of the NDF content to liver Cu retention was poorly correlated (r^2 = 0.0454; y= - 0.0004x + 0.148). Fig. 8.1, however, indicates that the reduction in liver Cu retention was often associated with the increase in NDF content.

The exception was dried grass pellets vs. maize silage and grass silage vs. fermented WCW where, despite a higher fibre content in dried grass pellets and grass silage, but liver Cu status was higher in lambs fed dried grass pellets and grass silage forages possibly due to a higher Cu intake. Fig. 8.2 shows the relationship between Cu intake and liver Cu retention of the data obtaining from Chapters 3, 4, and 6. These results indicate that the regression

coefficient of the Cu intake to liver Cu retention was also poorly correlated (r^2 = 0.175; y=0.00729x). However, Fig. 8.2 indicates that liver Cu retention was increased by increase in Cu intake.

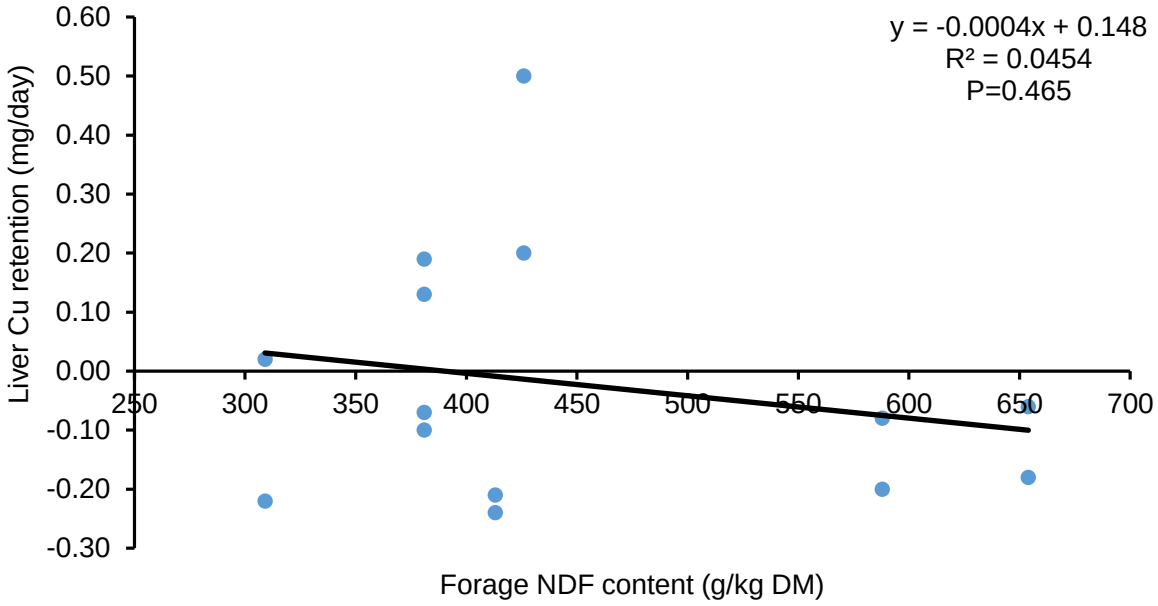


Figure 8.1. The relationship between the forage NDG content and liver Cu retention from sheep fed experimental diets from Chapters 3, 4, and 6.

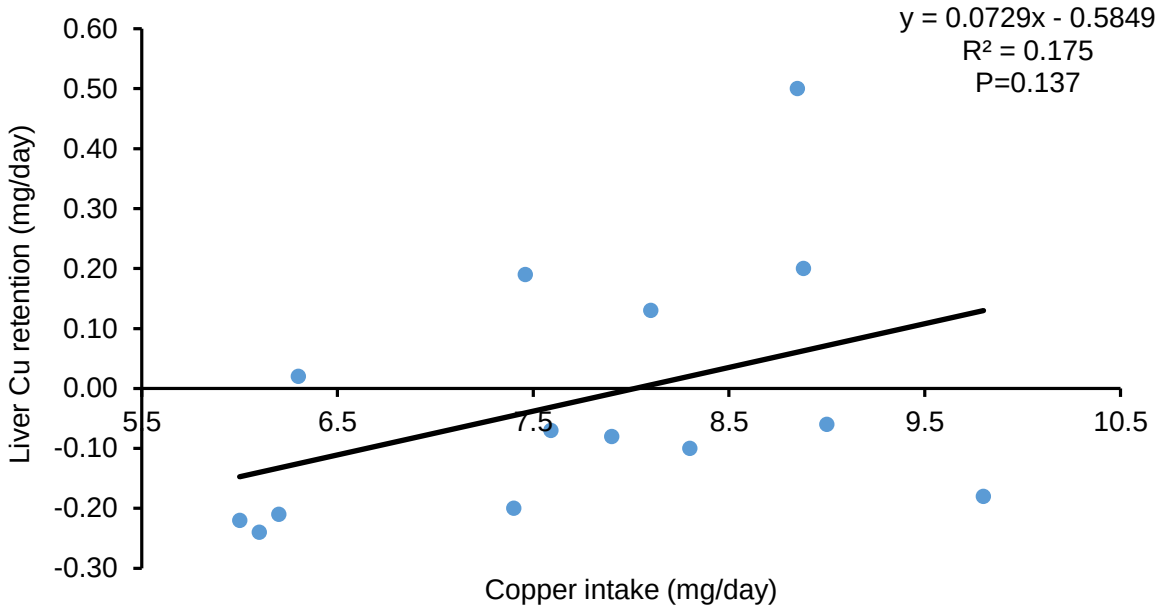


Figure 8.2. The relationship between the Cu intake and liver Cu retention from sheep fed experimental diets from Chapters 3, 4, and 6.

In addition, the smaller feed particle size in dried grass pellets may have resulted in an increased proportional outflow of particulate matter from the rumen (Thomson and Beever, 1979; Mason, 1990) reducing the residence time and hence increasing Cu availability due to avoiding expose to Cu antagonists present in the rumen (Suttle, 1983a).

In Chapter 5, the results demonstrate that *in vitro* gas production was higher in maize silage forage compared with grass haylage. Similarly, in Chapter 7, gas production *in vitro* of fermented grass silage forage was higher compared with artificial grass hay. Ibrahim *et al*. (1990) demonstrated that *in vitro* organic matter digestibility was higher in maize silage compared with wheat straw (661 and 405 g/kg) respectively. Gosselink *et al*. (2004) found a strong correlation (r^2 =0.90) between gas production and organic matter truly digested in the rumen of sheep for 12 fresh and conserved forages. As a consequence, the higher gas production in maize silage than grass haylage could indicate the higher digestibility of maize silage and hence more minerals may be released from silages than dried forages, as dried diets allow a considerable amount of organic matter to leave undigested from rumen may supply plentiful binding site for products of the CuxMoxS interactions and a decrease in Cu absorption (Suttle, 1991). The digestibility of forages in the Chapter 6 was not determined, but the application of urea has been shown to elevate DM digestibility of WCW (Ørskov *et al*., 1983). Adesogan *et al*. (1998) showed that the digestibility of organic matter content in the DM of urea WCW was higher compared fermented WCW, with mean values of 646 and 611 (g/kg) respectively. Similarly, Walsh *et al*. (2008) demonstrated that the DM digestibility of fermented WCW was lower that urea WCW. Moreover, Cu in feedstuffs has been reported to be mainly associated with the lignocellulose compartment (Ibrahim *et al*., 1990) and alkali treatment can dissolve the lignocellulosoic cross-linking of the cell (Hill and Leaver, 1999). Similarly, ammonia application has been reported to decrease the NDF fraction, due to the partial solubilisation of hemicellulose (Haddad *et al*., 1994). The NDF content in Chapter 6 was lower in urea WCW compare to fermented WCW (309.2 and 413. g/kg DM) respectively. Therefore, applying urea to the WCW possibly contributed to an increase feed digestibility and break down of plant cell wall and hence a greater Cu has been released into the rumen and increased Cu availability for absorption by the animal.

The difference in liver Cu status could be related to the effect of rumen pH and its subsequent effect on rumen micro-organisms and sulfide production. In Chapters 3, 4, and 6 higher liver Cu status in lambs fed dried grass pellets, maize silage, and urea WCW respectively were coupled with lower rumen pH in the lambs fed these forages. However, results from Fig. 8.3 indicates that the regression coefficient of the rumen pH to liver Cu retention was very poorly correlated (r^2 = - 0.012; y=0.1341x + 0.8119). Figure 8.3 also

indicates that liver Cu retention was slightly decreased with increase in rumen pH. Crosby *et al.* (2004) found that the lambs bedded on straw had a lower (15%) liver Cu concentration compared with those lambs housed on the expanded metal floor (which had access only to the concentrate diet with no additional roughage source). Crosby *et al.* (2004) suggested that the roughage intake by the straw group possibly would have elevated rumination and salivation, resulting in an increased rumen pH, which in turn, promote rumen sulfide producing ciliate protozoa and reduced Cu availability. A decline in ruminal or *in vitro* pH may be contribute to a reduction in total protozoal concentrations (Dehority and Orpin 1997; Dehority, 2005; Belanche *et al*., 2016). The effect of protozoa on Cu metabolism has been suggested to be through the reduction of sulfate and also degradation of S-containing amino acids in the anaerobic rumen conditions to form sulfide, which then interacts with Cu to produce insoluble a Cu-S complex (Dick *et al*., 1975; Spears, 2003; Suttle, 2010). For example, it was reported that rumen ciliate protozoa affects dietary Cu metabolism by reducing Cu absorption by up to 50% and decreases the incidence of Cu toxicity in fauna free (no rumen protozoa) sheep (Ivan *et al*., 1985). In addition, it was reported that the increase of protozoa population in fauna free sheep contributed to an increase in rumen sulfide production, decrease in Cu solubility, and liver Cu concentration compared to fauna free sheep (Ivan *et al*., 1991; Ivan and Entz, 2007). Moreover, low rumen pH has been reported to facilitate an enhanced sulfide absorption from the rumen (Bray *et al*., 1975), thereby, sulfide interacts with Cu to form insoluble Cu-sulfide possibly reduced. Therefore, the higher liver Cu status in this thesis, which was also paralled with a low rumen pH and fibre intake appears to confirm the adverse effect of the higher rumen pH and fibre content on Cu metabolism.

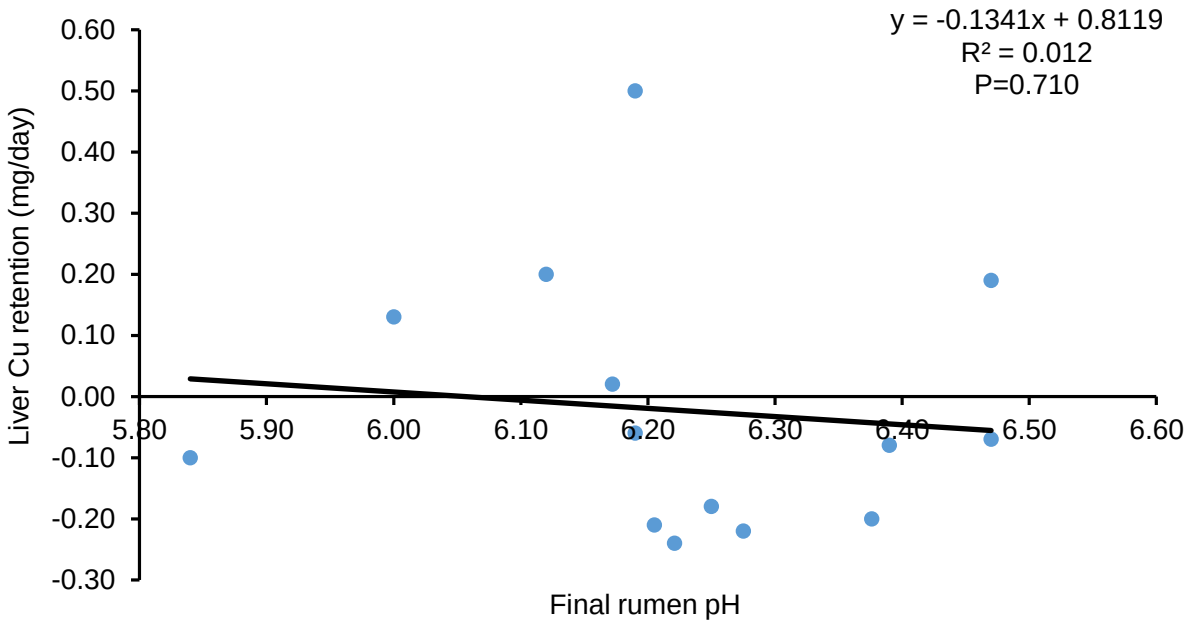


Figure 8.3. The relationship between the final rumen pH and liver Cu retention from sheep fed experimental diets from Chapters 3, 4, and 6.

The antagonist effect of additional Mo and S on reducing liver Cu status has been well researched in sheep (Dick, 1953; Suttle and Field, 1968; Suttle, 2012) and cattle (Sinclair *et al*., 2013; 2017). Small increase in herbage Mo and S has been reported to cause a substantial reduction in Cu availability (Suttle, 1983b). In Chapters 3, 4, and 6 lambs offered diets unsupplemented with Mo and S had a higher liver Cu concentration compared with the lambs fed diets supplemented with antagonists. Crosby *et al*. (2004) reported that dietary Mo at 5 mg/kg DM fed to Texel cross lambs resulted in a substantial decrease in liver Cu concentration. In ruminants two possible physiological sites of CuxMoxS interactions have been proposed; in the first site, primary interactions between Cu, Mo, and S are suggested to occur in the rumen and lower gut, by which insoluble triple complex Cu-thiomolybdate ($CuMoS_4$) or Cu-sulfide (Cu-S) are formed (Spears, 2003; Suttle, 1991; Gould and Kendall, 2011). Copper in both forms are believed to be not available for absorption and hence liver Cu reserves would become decreased to meet tissue requirement for cupric proteins such as Cp (Robinson *et al*., 1987). The second site is combined between gut and post-absorptive (systemic effect) and where the produced rumen thiomolybdates would be absorbed into the blood circulation. Therefore, for thiomolybdates to have a systemic effect on tissue Cu, they first must be absorbed (Suttle, 1991).

Thiomolybdates have been used as a treatment and prevention of Cu toxicity in sheep (Humphries *et al*., 1986; Kumaratilake and Howell, 1989; Goonerante, 2012). The mechanism by which thiomolybdate reduced liver Cu status has been suggested to be either directly by removing Cu from hepatocyte lysosomes of Cu-loaded sheep as indicated by the increased activity of β-glucuronidase enzyme in bile after intravenously administered tetra-thiomolybdate and increased biliary Cu excretion (Goonerante, 2012), or indirectly. The first indirect mechanism is that absorbed thiomolybdate in the blood stream binds with Cu in albumin and producing an excretable and slowly hydrolysis Cu-TM-albumin complex (Manson, 1986), serving as a pool of slowly released Cu, thus resulting in delay in transfer of Cu to tissues including liver (Goonerante *et al*., 1989a; Suttle, 1991; 2010). The second, upregulation of hepatocyte ATP7B, which is responsible for Cu efflux from the hepatocyte to the bile for excretion (Linder, 2010; Wadwa *et al*., 2014). In cows after feeding a diet supplemented with Mo and S in combination with the organic Cu, the expression of Atp7b tended to be greater compared with cows fed a diet unsupplemented with antagonists (Sinclair *et al*., 2013). The third mechanism is that, thiomolybdates have been suggested to cause impairment of entero-hepatic recycling of Cu (Suttle, 2010), possibly due to absorbed thiomolybdate in the blood binding to Cu with a such strong affinity that Cu in Cp protein is not being recycled back to the liver and broken down as a result half-life time of Cp (2-3 d; Linder, 1991) may be altered, resulting in a decreasing liver Cu (Williams, 2004).

However, in this thesis in Chapters 3 and 6 blood Cu status such as Pl-Cu concentrations, and Cp activity were not affected by dietary addition of antagonists, except in Chapter 4, where Pl-Cu concentration and Cp activity were reduced. This reduction in Cp activity was not clear whether it was due to a direct effect of rumen thiomolybdates (Suttle and Field, 1968; Zhou *et al*., 2016), or due to systemic effect of thiomolybdates (Williams, 2004; Mackenzie *et al*., 2008). Robinson *et al*. (1987) reported that addition of Mo (6.6 mg/kg DM) and S (4 g/kg DM) the lambs diet resulted in a significant reduction in liver Cu concentration, Pl-Cu concentration, and Cp activity, although, by increasing dietary Mo supplementation to 11 mg of Mo/kg DM the reduction in liver Cu concentration was accompanied by a decrease in Cp with an increased in Pl-Cu concentration. Robinson *et al*. (1987) suggested that the reduction in liver Cu concentration, which was accompanied by a decrease in Pl-Cu and Cp in the case of 6.6 mg of Mo/kg DM may potentially be attributed to the gut effect of thiomolybdates, but at the higher level of additional Mo (11 mg/kg DM), where Pl-Cu concentration increased, possibly due to the systemic effect of thiomolybdate. Williams (2004) and Mackenzie *et al*. (2008) also supported the hypothesis of a systemic effect of thiomolybdate antagonism on Cu metabolism, when the addition of Mo at 5 -10 mg/kg DM resulted in an increased Pl-Cu concentration and decrease in both liver Cu concentration and Cp activity. The formation of systemic Cu-thiomolybdate complexes has been suggested to be most probably responsible for the decrease in liver Cu status and increase in Pl- Cu concentrations in ewes receiving Mo and S in comparison to the respective control ewes (De Plessis *et al*., 1999b).

The ratio of Cp:Pl-Cu has been suggested to be more beneficial compared with Pl-Cu concentration in ruminants for detecting whether thiomolybdates are being absorbed into the blood or not (Mackenzie *et al*., 1997; Kendall *et al*., 2000; Mackenzie *et al*., 2001). Williams (2004) and Mackenzie *et al*. (2008) showed that the addition of Mo significantly reduced Cp:Pl-Cu ratio, whereas, in Chapter 4 and addition of antagonists had no effect of Cp:Pl-Cu ratio. Moreover, Suttle (2010) and Sinclair *et al*. (2017) suggested a dietary Cu:Mo ratio (1:1) as a threshold as indicative of TM being absorbed from rumen into the blood stream and causing a systemic impairment of Cu-containing enzymes. In Chapter 4, the dietary Cu:Mo ratio was reduced by supplementation with molybdenum in both diets from approximately 7.3:1 to 2.2 :1, which was greater than (1:1). Although, in studies conducted by Robinson *et al*. (1987), Williams (2004), and Mackenzie *et al*. (2008) the Cu:Mo ratio was 1:1 or less. The reduction in Pl-Cu concentration and Cp activity (in Chapter 4) appears to suggest that possibly a greater gut effect of thiomolybdate, however, a systematic effect of thiomolybdates cannot be ruled out.

In Chapters 5 and 7 the majority (over 85%) of Cu, Mo, and S was found to be associated with the solid phase. The forages that were used in Chapter 5 were the same forages fed to lambs in Chapters 3 and 4. The high distribution of Cu and thiomolybdate (Mo) in the solid phase of the rumen has been suggested to be facilitated by the formation of Cu-thiomolybdate complexed in the rumen, as the association of TM with the solid phase possibly imparts some stability for the thiomolybdates in the rumen and escape from hydrolysis in the abomasum due to a low pH (Gawthorne *et al*., 1985; Suttle, 1991; Gould and Kendall, 2011). Moreover, in Chapter 5 the addition of Mo and S generally reduced the distribution of Cu in the supernatant fraction possibly due to increased Cu associated with the solid phase. Likewise, Allen and Gawthorne (1987) also reported that addition of antagonists increased Cu associated with undigested feed particles, protozoa, and bacteria of rumen digesta and using TCA and neutral detergent solution (NDS) to break down complexes, Allen and Gawthorne (1987) reported that 29-78% of the Cu remained unextracted in samples supplemented with antagonists compared with 1-6% for samples from sheep unsupplemented with antagonists, this implying that Cu was bound to the high molecular protein. Price and Chester (1985) showed that rumen digesta from sheep fed dried grass supplemented with 11 mg of Mo/kg DM given to Cu-deplete rats had a poor capacity to replete the activity of cytochrome oxidase in the intestine. Moreover, Suttle and Field (1983) reported that additional tetra-thiomolybdate in sheep diets reduced Cu absorption. These findings suggest that thiomolybdates cause Cu to be irreversibly bound to a high solid phase and thus decrease Cu absorption and deplete liver Cu status in Chapters 3, 4, 6. Therefore, results from this thesis confirms the hypothesis suggesting an intra-ruminal formation of insoluble Cu-thiomolybdate complexes, which are poorly absorbed and hence reduce Cu status.

The degree to which thiomolybdates are produced in the rumen and its impact on how Cu availability is changed by basal diets being offered, although understanding of the mechanisms remains poor (Ivan, 1993; Suttle, 1986; Sinclair and Mackenzie, 2013). Results from Chapters 3 and 4 indicated that there was no significant effect of an interaction between forage type and Cu antagonists. However, in Chapter 6, liver Cu retention was not affected by the addition of Mo and S in growing lambs fed fermented WCW, while in lambs fed urea WCW or grass silage liver Cu retention was markedly reduced by the addition of antagonists. The lower inhibitory effect Mo and S on liver Cu retention in lambs fed fermented WCW compared with grass silage potentially related to the lower rumen pH in fermented WCW than grass silage, as was observed in Chapter 6. Suttle (1991) discussed the effect of rumen pH on reducing the inhibitory effect of Mo and increase Cu availability. It is stated that feeding highly fermentable carbohydrate content diets such as whole grains to sheep did not accelerate the rate of liver Cu depletion, when dietary Mo increased from

2.5 to 5 mg/kg DM possibly due to the carbohydrates lowering the rumen pH, resulting in increased a breakdown of thiomolybdates (Suttle, 1991). More recently, Sinclair *et al*. (2017) demonstrated that the reduction in liver Cu retention as a result of addition of Mo and S was greater in cows fed grass silage compared with maize silage. In Chapter 5, the *in vitro* pH of fermented maize silage was lower compared to grass silage. Moreover, Wang *et al*. (1988) reported that 13-14 weeks were required to induce diarrhoea in steers fed Mo rich silage, containing 35 mg/kg DM, while cattle grazing pasture containing a similar Mo concentration exhibited immediate diarrhoea or ''teartness'' (Ferguson *et al*., 1943).

Liver Cu concentration from Chapters 3, 4, and 6 are presented in Table 8.3 and all values were within the normal range of 100-400 mg/kg DM (NRC, 2005) in both unsupplemented or Mo and S supplemented lambs. Using data obtained from Chapters 3, 4, and 6, Fig 8.4 illustrates the liver Cu retention at the end of each respective study for three different breeds. These results indicate that Texel cross breed lambs had a positive liver Cu retention and gained approximately 2 mg of Cu/d, whereas, Swaledale and Scottish Blackface lost 0.05 and 0.15 mg of Cu/d respectively. The mean of Cu intake in Texel cross breed (Chapter 3) was 8.2 mg of Cu/d similar to the Cu intake in Swaledale breed (Chapter 4) (8.8 mg of Cu/d), with the lowest Cu intake in Scottish Blackface lambs (Chapter 6) (6.7 mg of Cu/d). In addition, initial liver Cu concentration was lower in Texel cross compared with other breeds (Table 8.3). Therefore, these differences in liver Cu retention is likely due to the breed effect, as the difference in Cu intake between breeds was less pronounced. Recently, Sefdeen (2017) reported that over a period of 10 weeks of feeding a diet containing 13.6 mg/kg DM of Cu, liver Cu retention in Texel cross lambs was higher compared with Swaledale, with mean values of 1.89 and -1.12 mg of Cu/d respectively. The effect of genetic variation on Cu metabolism in sheep is well recognised (Suttle *et al*., 2002). Breeds such as Scottish Blackface and Welsh Mountain are generally recognised as more susceptible to Cu deficiency compared to Texel, North Ronaldsay and Suffolk that are more prone to Cu toxicity (Suttle, 2010). It has been shown that liver Cu retention in Blackface x Texel Scottish lambs to be higher than pure Scottish Blackface lambs (13.7% and 5.6% of ingested Cu respectively (Woolliams *et al*., 1982). These differences were attributed to the difference in Cu absorption, as dietary Cu absorption was found different between Scottish Blackface and Welsh Mountain (4.3% vs. 7.3%) respectively (Woolliams *et al*., 1983).

Table 8.3. Initial and final liver Cu concentration of growing lambs used in different Chapters.

Chapter	Breed	Initial liver Cu concentration (mg/kg DM)	Final liver Cu concentration (mg/kg DM)
3	Texel cross	173.6	241.0
4	Swaledale	268.4	177.0
6	Scottish Blackface	323.7	159.7

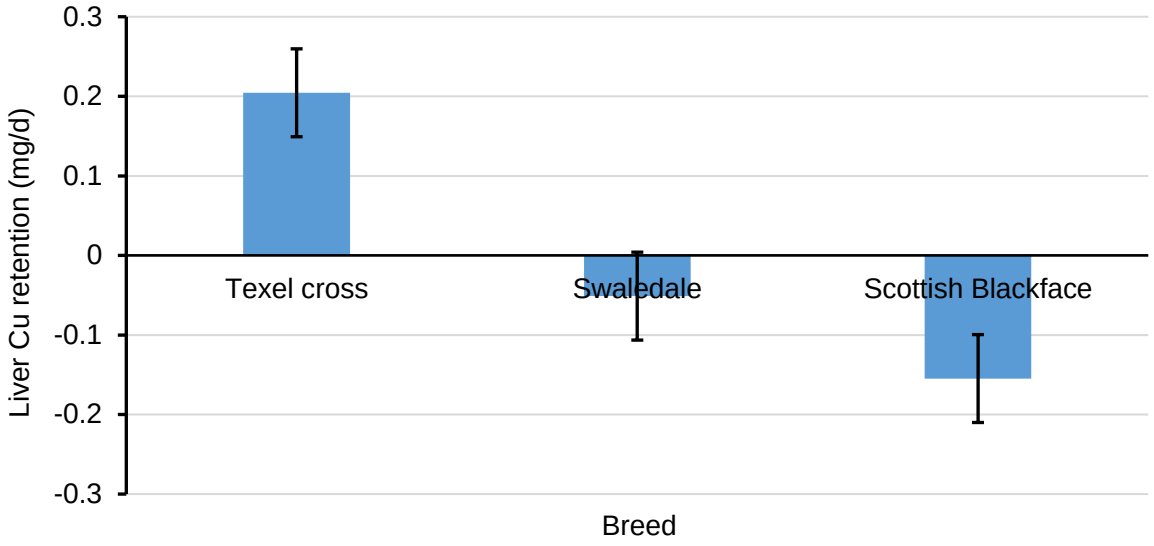


Figure 8.4. Liver copper retention from sheep fed experimental diets from Chapters 3, 4, and 6.

8.5. Conclusions and further work

Results presented in this thesis show some novel findings with respects to the interaction between forage type and Cu metabolism. The effect of forage type on Cu metabolism has been confirmed, where liver Cu status was higher from forages that allowed a more acidic rumen environment either due to their higher NSC, lower NDF or rumen outflow rate. However, the mechanism of this effect was not clear in this thesis, but would be of interest for future studies. In addition, preservation of WCW as a urea WCW would be more beneficial than fermented in terms of increasing Cu availability. Alterations in liver Cu status and blood Cu due to the addition of Mo and S also have been observed. These findings not only confirm the hypothesis and findings from previous authors within the literature, but have generated ideas for future work to determine whether the antagonism effect of Mo and S are due to a gut effect or systemic effect of absorbed thiomolybdate. The lower impact of antagonists on liver Cu status in the FWCW compared with UWCW, when both had a similar rumen pH generate ideas. At high dietary Mo and S levels preserving WCW as FWCW than UWCW may be more beneficial in terms of Cu metabolism. In addition, the lower rumen pH from FWCW and also lower response to Cu antagonists compared with grass silage, suggesting that a more acidic rumen environment may alleviate the adverse effect of thiomolybdates on Cu availability. Additional studies are also required to study the effect of forage type on rumen pH and its influence on the rumen thiomolybdate formation and Cu metabolism. The lower liver Cu status Swaledale and Scottish Blackface lambs than Texel cross, confirms the genetic variation between breeds in terms of Cu metabolism. Taking breed of sheep into consideration would be important during feed formulation in order to determine Cu requirements and avoid Cu toxicity or deficiency.

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