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EFFECT OF DIETARY IRON ON COPPER METABOLISM OF SHEEP

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Effect of Dietary Iron on Copper Metabolism of Sheep

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Abstract

A series of experiments were carried out to investigate the effect of dietary iron (Fe), sulphur (S) and breed on copper (Cu) metabolism and performance of growing lambs. In the first study (presented in Chapter 3), a basal diet containing 487.6 mg/kg DM Fe and 4 g/kg DM S, supplemented with 0, 250, 500, and 750 mg/kg DM Fe were given to weaned Texel lambs to investigate effects on Cu status, performance and nutrient balance. There was no effects of dietary treatments on lamb performance. Dietary supplemental Fe significantly reduced liver Cu storage but increased liver Fe storage of lambs with the greatest effect observed at 750 mg Fe/kg DM. Dietary supplemental Fe in this study had no effect on other Cu parameters measured. It was also observed that Fe supplementation significantly increased plasma Fe concentration but had no effect on other minerals concentration. Supplemental Fe significantly increased urinary Mo output in all lambs receiving Fe but had no effect on the urinary output of other minerals. In the study presented in Chapter 4, three levels of supplemental S (0, 1.5 and 3 g/kg DM; basal S = 2.89 g/kg DM) and two levels of supplemental Fe (0 and 800 mg/kg DM; basal Fe = 302.3 mg/kg DM) were given to weaned lambs to investigated the effect of different levels of S with or without Fe on Cu metabolism. Dietary Fe significantly reduced the plasma and liver Cu concentration of lambs after 12 weeks of supplementation. Liver and plasma Fe concentration increased significantly in groups given Fe supplemented diets. Plasma Mo increased significantly in lambs fed supplemental Fe. Dietary S had no significant effect on liver, plasma or the biliary Cu, Fe, and Mn concentration, but significantly increased biliary Mo concentration in lambs. Ceruloplasmin activity (Cp) and Cp:Pl-Cu ratio increased in Fe supplemented lambs but Cp activity decreased in groups given S supplemented diets. In the last series of studies (presented in Chapter 5), the effect of dietary Fe and breed of sheep on Cu metabolism was investigated. Weaned Scottish Blackface and Texel lambs were fed a basal or Fe supplemented diets (800 mg Fe/kg DM; basal Fe= 257.5 mg/kg DM). Dietary Fe reduced plasma Cu but increased plasma Fe and Mo concentration. Scottish Blackface had higher plasma Cu concentration than Texel lambs, but Texel lambs had higher plasma Mo concentration than Scottish Blackface lambs. In the second part of this study (experiment 2), weaned Swaledale and Texel lambs were fed basal or Fe supplemented diet (368.7 mg/kg DM; basal Fe= 532.4 mg/kg DM). Supplemental Fe significantly decreased liver Mo but had no effects on other the liver minerals concentration. Texel lambs retained significantly more Cu in their livers than Swaledale lambs after ten weeks feeding. Plasma Cu and Zn concentration decreased significantly in the lambs fed Fe supplements. These studies confirm that Fe is a potent Cu antagonist in sheep and highlights the differences between sheep breeds in their Cu metabolism and responses to antagonists.

Table of Contents

List of Tables

List of Figures

Part of the work in this thesis has appeared previously:

Sefdeen, S.M., Mackenzie, A.M., Wilkinson, R.G. 2014. The effect of iron supplementation on hepatic copper content of growing lambs. In: Proceedings of the British Society of Animal Science (Annual Meeting), Nottingham, p 031.

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Author Declaration

This thesis has been composed entirely by myself and it has not been accepted in any previous application for a degree. The work, of which it is a record, has been done by myself.

Sherwan Mostafa Sefdeen

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CHAPTER 1

Literature Review

1.1. Introduction

Micro or "trace" minerals differ from macro minerals depending on the amount required in the diet, and on metabolic needs of the animal (NRC, 2007). Trace minerals are required at concentrations less than 100 mg/kg diet and their concentration is less than 50 mg/kg in the body (McDonald *et al.*, 2011), while macro minerals are required at concentrations greater than 100 mg/kg diet (McDowell, 2003). The term 'essential mineral element' is restricted to a mineral that has been proven to have a metabolic role in the body (McDonald *et al*., 2011).

According to the NRC (1996), 15 trace minerals are referred to be essential in animal nutrition, 10 of which are considered important and need to be supplemented when the diet dose not supply sufficient amounts, including chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn). Additional trace minerals, including arsenic (As), boron (B), lead (Pb), silicon (Si), and vanadium (V), have also been identified as important; however, no critical function has been identified in animals (NRC, 1996). Other researchers have identified a similar list of essential trace minerals (for virtually all animal species) and have increased the number of essential trace minerals to 18 due to the inclusion of fluorine (F), lithium (Li), and tin (Sn) (McDowell, 2003). The requirements and the maximum dietary concentration of some important minerals in sheep diets are present in Table 1.1.

For normal tissue growth, homeostasis, enzyme function, and cell regulation, it is important to maintain essential trace minerals concentration within narrow concentrations in the body (Suttle, 2010). When appropriate trace mineral homeostasis is maintained (beside other nutrients), optimum growth, health, and productivity of domestic livestock can be guaranteed (Underwood and Suttle, 1999). When homeostasis is not successful, toxicity or deficiency can result (McDowell, 2003).

Trace mineral deficiencies can be either primary or secondary (Smart *et al*., 1981). Primary deficiency is due to the lack of a trace mineral in the diet, while a secondary deficiency is due to inhibition of homeostasis as a result of the presence of one or more antagonistic minerals that reduce the availability of the trace mineral (Ullrey *et al*., 1977).

Most of the essential trace minerals are present in adequate concentrations in commonly used feedstuffs (NRC, 1996), but mineral deficiencies usually occur due to the inability of the animal to absorb or utilize a trace mineral as a result of the presence of the antagonist minerals in the gastrointestinal tract (Suttle, 2010).

Table 1.1. Macro-mineral and micro-mineral requirements of sheep (NRC, 2007)

Minerals	Requirements
Macro-mineral (g/kg DM)	
Na	0.5 - 0.6
Cl	0.4 - 0.5
P	1.6 - 3.8
K	4.2 - 4.8
Mg	0.9 - 1
S	1.7 - 1.8
Ca	2 - 8.2
Micro-mineral (mg/kg DM)	
Co	0.1 - 0.2
Cu	4.1 - 6.1
I	0.5
Fe	28.4 - 82.6
Mn	15.1 - 22.9
Se	0.1 - 0.2
Zn	21.8 - 38.9
Mo	0.5

Copper deficiency in ruminants is problematic in many areas of the world (Underwood, 1977). Dietary Cu antagonist such as Mo, S, and Fe can be found at high concentrations in soils, feedstuffs, or water that can greatly reduce Cu balance of ruminants (Underwood, 1977; Kerr *et al*., 2008; Gould and Kendall, 2011). Calcium, Cd, Zn and Ag are other Cu antagonists that can reduce Cu absorption when present in a high concentration in the diet (Suttle, 2010; Underwood, 1977). Iron is a potent Cu antagonist that decreases body Cu stores in sheep and cattle fed diets high in Fe concentration (Standish *et al*., 1971; Campbell *et al*., 1975; Bremner *et al*., 1978; Williams, 2004).

Yet little is known about the mechanism by which Fe alters Cu balance of sheep. Iron effects are not restricted to ruminant animals only, reductions in the tissue Cu concentrations and the bioavailability of Cu have been reported in the rats given diets high in Fe (Bremner and Price, 1985). It has been suggested that antagonistic effect of Fe in ruminants arise from its interaction with ruminal sulphide, with consequent trapping of the sulphide and increase in its ability to reduce absorption of Cu from the small intestine (Suttle *et al.*, 1984). The aims of the current experiments were to further investigate and

enhance the knowledge behind the antagonistic effect of Fe and S on Cu status and metabolism of sheep of different breeds.

1.2. Chemical properties of copper

Copper is a transition mineral with an atomic mass of 63.54 daltons and a density of 8.94 g/cm^3 (Pollard *et al*., 2007). Copper's atomic number is 29 and is the first element of the IB group (group 11) of the periodic table (Galasso, 2016). Copper has two important stable isotopes, ^{63}Cu and ^{65}Cu, with the natural abundances of 69.2 and 30.8%, respectively (Wiederhold, 2015). These Cu isotopes are usually used as Cu metabolism tracers in animals (Wapnir, 1998; Georgopoulos *et al*., 2001). There are other Cu radioisotopes and most with half-lives of seconds or minutes (De Lima, 2011) which are not important in animal physiology or metabolism. Copper occurs naturally in the environment as a free metal or associated with other elements that comprise different compounds (Georgopoulos *et al*., 2001). It is present in one of four oxidation statuses: Cu^0 (Cu metal; small extent), Cu^{1+} (cuprous ion), Cu^{2+} (cupric ion, predominant), and Cu^{3+}, but the trivalent state is rare. Cu^0 is stable, but it gets dissolved in strong acids, for example sulphuric acid (H_2SO_4) or nitric acid (HNO_3) (Georgievskii *et al*., 1982; Georgopoulos *et al*., 2001). The cuprous compounds are commonly colourless and oxidise rapidly in aqueous solution to the Cu^{2+} form with blue or green colour which is the most important state (NRC, 2005).

1.3. Sources of copper in nature

Copper occurs naturally in rock, soil, sediments, water, feedstuff, and air. Table 1.2 and Table 1.3 shows the Cu concentrations of some feed ingredients, tissues, soil, and water. Copper's concentration averages 50 mg/kg (ranges from 45 to 70 mg/kg) in the earth's crust (Aubert and Pinta, 1977). Copper concentration in soil depends on the geology and it typically varies from 2 - 50 mg/kg DM. However, higher concentrations can also be found in some soils (Oorts, 2013). In EU soils, the average Cu concentration is 31.1 mg/kg DM (Heijerick *et al*., 2006). The concentration of soil Cu in England and Wales is presented in Table 1.2. Pastures and forages are the main Cu source for ruminants and their Cu content is influenced by soil genesis, condition of soil, fertilizer application and plant factors (Grace and Clark, 1991).

Table 1.2. Copper content of waters and soils from different texture classes of England and Wales (Linder, 1991; Zhao et al., 2007; Guo et al., 2010)

Item	Copper content
Fresh water (µg/l)	0.1 - 1.0
Sea water (surface) (µg/l)	1.0
Sea water (1000 m) (µg/l)	100.0
Sandy (mg /kg DM)	7.4
Coarse loamy (mg /kg DM)	15
Coarse silty (mg /kg DM)	19
Fine silty (mg /kg DM)	19
Fine loamy (mg /kg DM)	19
Clayey (mg /kg DM)	23
Peaty (mg /kg DM)	15

1.3.1. Copper in water and soil

In water Cu can exist in 3 categories: particulate, colloidal, and soluble, but water Cu bioavailability is low due to Cu adsorption to suspended particles (WHO, 1998). Besser *et al*. (1996) reported that sediment Cu seems to have a low bioavailability due to its capability to react with acid volatile sulphides forming insoluble complexes in the water. Physio-chemical processes may result in Cu dissolving in water or associating with particles and colloidal matter; Cu in particles consist of precipitates, insoluble organic complexes and adsorbed Cu to clay and to other mineral solids (Georgopoulos *et al*., 2001). The Cu released into the water is usually in particulate forms that tend to settle out, precipitate, adsorbed to other minerals present in the water or clay sediments that lead to decrease water Cu concentration downstream (NRC, 2005). Due to the low level of Cu in clean water (0.15 µg Cu/L and 1.0 to 20 µg Cu/L in sea and fresh water, respectively) and because of its low bioavailability (WHO, 1998), water Cu concentration does not count as an important source of Cu in animal nutrition.

The soil's Cu content normally differs from one location to another, for example in the US, soil Cu concentration is greatly different between regions and averages 25 mg Cu/kg DM. The Cu concentration is higher in areas closer to Cu mining smelting activities (Mohamed and Antia, 1998; WHO, 1998). In New Zealand, Longhurst *et al*. (2004) found that soil Cu concentration ranged from 8.7 to 32.3 mg/kg when 312 sites samples were analysed. In the UK, the average Cu concentration of the surface soil ranges from 7.4 to 23 mg/kg DM in sandy and clay soils, respectively (Zhao *et al.*, 2007). The top few centimetres of soil contains the majority of soil Cu which is strongly adsorbed to soil particles and organic materials (Hickey and Kittrick, 1984). High organic materials in the soil may reduce Cu

availability to plants and to leach it out from the soil (Georgopoulos *et al*., 2001; McBride, Richards *et al*., 1997).

The majority of Cu present in the soil is complexed with organic matter and up to 99% of soil Cu, at pH 5.15, was extracted from high organic containing soil when treated with a solution containing 0.5 M EDTA (Sauvé *et al*., 1996; Palma and Ferrantelli, 2007). Therefore, the mobility of organically complexed Cu is rather low and its bioavailability is limited to plants due to the tightly binding of Cu^{2+} with the organic matter of the soil more than to the other divalent transition metals (McBride *et al*., 1997; Mohamed and Antia, 1998). At acidic pH, dissolved Cu increases because of its weaker adsorption to organic matter molecules present in the soil, and subsequently Cu concentration of free Cu ion increases. Soil physicochemical parameters, such as soil texture, pH, and organic matter content, interact to determine the adsorption process of metals in soils, consequently affecting their availability to plants (Ginocchio *et al*., 2002).

Most minerals are readily available to plants at about neutral soil pH (pH = 6.5 - 7.5), but decrease in both acidic and alkaline soils (Grime, 1973; Gould and Walker, 1999; Pausas and Austin, 2001). Plant species diversity is lower in most acidic soils (Dupré *et al*., 2002) because the essential nutrients such as: Ca, Mg, K, PO_4, and Mo are in unavailable forms to plants that develop nutrient deficiency (Larcher, 2003). Generally, the greatest portion of the colloidal substance of soils, such as oxides of Mn, Fe, and Al, silicate clays and humus, adsorbs Cu^{2+} strongly in an increasing manner with increasing soil pH and for this reason, farmers are able to apply high amounts of Cu salts to organic soils over a long period without observing any toxicity effects to their crops (Mohamed and Antia, 1998). However, this practice is not common in the UK. Increases in the soil pH by lime application to reduce soil acidity and increase crop production, leads to an increase in the binding affinity of Cu to organic and inorganic matter of the soil (Conrad *et al*., 1984; McDowell, 1996; Mengel *et al*., 2001; Oorts, 2013).

When soil pH increases, Cu deficiencies may develop in plants and Mo toxicity can arise due to an increases Mo uptake by the plant and subsequently by the animals (Suttle and Jones, 1989). Lime application increases soil pH that increase Mo uptake by plants (McDowell, 1996; Mengel *et al*., 2001; Oorts, 2013). The trace elements availability to plants is controlled by the total concentration of minerals in the soil and their chemical form (Ma and Rao, 1997).

In highly acidic soils, certain ions (Al^{3+}, Cu^{2+}, Fe^{3+}, Mn^{2+}) may rise to toxic levels in the majority of plants (Foy, 1992; Kinraide, 1993; Silva, 2012). Moreover, acidic soils have a high capacity of cation exchange, and promote leaching of nutrients (Johnson, 2002). Soil pH is the principal factor controlling the free ions concentrations of the soil solution (Lofts

et al., 2004). It has been found that concentrations of all the elements in ryegrass decrease as a simple linear function of increasing soil pH and this response was consistent across the range of pH values, 4.2 - 7.0, measured (Smith, 1994). Soil drainage, beside soil pH, is also an important factor that affects the solubility and availability of many trace elements such as Mn and Mo, increasing in poorly drained soils (Jiang *et al*., 2015). This could be due to the effect of redox potential on the availability of trace elements of variable valency, the combining power of an element, and to the larger quantities of organic matter in the solution under anaerobic conditions (Thornton, 1983).

1.3.2. Copper in feedstuffs

Copper is found naturally in all feedstuff including forages in different concentrations (Table 1.2). Concentration of forage Cu can differ greatly depending on the geographical region (Table 1.4), plant species, maturity stage, number of cuts, climate and seasonal changes, soil properties and fertility, and soil pH as discussed previously (Section 1.3.1). Application of high N fertilizer also has been found to reduce Cu content of the crops (Brennan, 1994; Givens *et al*., 2000; Ginocchio *et al*., 2002).

Normally, Cu concentration in various plant tissues ranges from 2 to 50 mg/kg DM (Marschner, 2012). The concentration of Cu in leaves ranges from 5 to 20 mg/kg DM (Baker and Senef, 1990), and can vary among plant species and varieties. The average Cu concentration of forages is 6.1 mg/kg DM (1278 samples), and approximately half of these samples had lower Cu content than the recommended minimum Cu requirements of ruminants (Minson, 1990). Similarly, in British Colombia 54% of the analysed forage samples (7 types) contained less than 5.7 mg Cu/kg DM (Miltimore and Mason, 1971). In a study conducted to determine the concentration of macro- and micro-mineral (including Ca, P, Mg, K, Na, Co, Cu, I, Fe, Mn, Se and Zn) of haylage samples from 124 farms in Sweden and Norway, Zhao and Müller (2015) found that the average mineral concentrations (SD) of the samples were as follows: Ca, 5.3 (3.41); P, 2.7 (0.80); Mg, 1.8 (0.76); K, 21.8 (7.44); Na, 0.3 (0.61) g/kg DM; and Co, 0.09 (0.150); Cu, 4.9 (1.61); I, 0.25 (0.461); Fe, 194 (288.9); Mn, 85 (49.3); Se, 0.03 (0.054); and Zn, 23 (9.5) mg/kg DM.

Table 1.3. Copper, iron, and S concentration in different animal feeds (mg/kg DM) (Sauvant et al., 2004)

ID	Cu	Fe	S	Mo
Barley grain	9 ±5	158 ±14	1.3	0.44
Maize grain	2 ±1	32 ±11	1.1	0.41
Oat grain	3 ±1	106 ±54	1.8	0.83
Sorghum grain	4 ±2	58 ±58	0.9	1
Soft wheat grain	5 ±1	47 ±14	1.5	0.46
Wheat bran	17 ±25	143 ±67	1.9	1.4
Maize distillers	10	105	3.2	1.7
Maize gluten meal	11 ±4	100 ±58	5.8	0.82
Maize bran	2	26	1.1	NA*
Maize germ meal, expeller	13	218	2.2	NA
Barley rootlets, dried	10	278	3.3	1.1
Brewers' dried grains	18 ±5	120 ±21	2.8	1.3
Cottonseed, full fat	10 ±1	63 ±32	2	1.5
Faba bean, coloured flowers	12 ±2	59	2.4	0.63
Faba bean, white flowers	11	73	1.8	0.63
Linseed, full fat	12	148	2.7	0.2
Lupin, blue	5	61	2.3	2
Lupin, white	4	24	2.5	2
Pea seed	7 ±1	92 ±29	2.0	2
Rapeseed, full fat	3	216	3.3	NA
Soybean, full fat, extruded	34	146	2.8	4
Soybean, full fat, toasted	34	143	2.8	4
Cottonseed meal, crude fibre 14-20%	10	184	3.3	3
Groundnut meal, detoxified, crude fibre <9%	17	335	3.2	1.7
Groundnut meal, detoxified, crude fibre >9%	15 ±1	516 ±15	3.1	2
Linseed meal, expeller	18	331	3.6	0.52
Linseed meal, solvent extracted	19	291	3.6	1.0
Palm kernel meal, expeller	21 ±9	534	2.2	0.40
Rapeseed meal	7 ±6	172 ±44	5.9	1.6
Soybean meal	18 ±7	283 ±15	4.0	4
Cassava, starch 67%	4	15	2.7	0.05
Sweet potato, dried	5	27	0.7	NA
Beet pulp, dried	5 ±2	601 ±31	2.4	0.67
Brewers' yeast, dried	47	97	4.3	1.1
Citrus pulp, dried	3 ±2	71 ±28	1.1 ±0.9	0.19
Molasses, beet	13	117	4.2	0.26
Soybean hulls	8 ±3	580 ±25	1.1	1.2
Alfalfa, dehydrated, protein < 16% DM	9	315	2.4	1.4
Alfalfa, dehydrated, protein 22-25% DM	7 ±1	309	2.7	1.4
Grass, dehydrated	7 ±1	525	1.0	2
Wheat straw	3	171	1.0	1.2
Milk powder, skimmed	3	7	3.0	0.24
Milk powder, whole	1.5	7	2.4	0.4
Whey powder, acid	1.6	9	4.7	5

* Values are not determined

In Latin American, forage Cu content is high with an average of 9 mg/kg DM, from 2615 samples, (McDowell *et al.*, 1977). In Kenya, forage Cu content range from 4 - 12.2 mg/kg DM, with an average of 7.4 mg/kg DM (Howard *et al.*, 1962). While in Scotland, hay and silage samples (132 and 146, respectively) have been analysed and observed that hay samples contain lower Cu concentrations compared with silage samples, 7.1 vs. 9.6 mg Cu/kg, respectively (Hemingway *et al.*, 1968).

Different plant species contain different levels of Cu, for example temperate legumes have a higher Cu (7.8 mg/kg DM) concentration compared with temperate grasses (4.7 mg/kg DM) (Hopkins *et al.*, 1994; Minson, 1990). Usually legumes contain less Cu than grasses in the tropical areas 3.9 vs 7.8 mg/kg DM, respectively (Minson, 1990). Leaves of temperate grasses containing an average of 35% more Cu compared with stems; variation between plant parts in Cu content depends on the age and maturity stage of the plant (Davey and Mitchell, 1968). There is also a large variation recorded in the Cu content of different feed ingredients or crop plants as shown in Table 1.3.

Table 1.4. Micro-mineral content in forages (minimum and maximum values) various studies (mg/kg DM) (source: Zhao and Müller, 2015)

ID	Jóhannesson *et al*. (2007) Iceland	Lindström *et al*. (2013) Sweden	MacPherson (2000) Northern Europe	Pirhofer-Walzl *et al*. (2011) Denmark (grasses and legumes)	Zhao and Müller (2015) Sweden and Norway
Mn	40–550	20–68	1–2670	44–83	12–364
Co	0.041–2.01	0.005–0.044	<0.01–1.26	NA*	0.01–1.20
Cu	4–16	2.2–8.5	4.0–8.2	4–11	1.8–11.0
Fe	57-1379	20–91	73–154	51–93	44–1991
Zn	14–85	13–40	3–300	18–29	13–96
I	NA	NA	0.09–0.42	NA	0.14–3.93
Se	0.006–0.096	NA	<0.05	NA	0.03–0.28

*Values are not determined

1.4. Copper functions

Copper is an important trace element required in the structure of over 300 proteins (Table 1.5). Deficiency of Cu impairs the function of Cu containing enzymes, and subsequently affects vital physiological processes in the body (Underwood, 1981; NRC, 2001; Bonham *et al*., 2002; Suttle, 2010). Copper containing enzymes function in fundamental processes such as energy metabolism, Fe utilization, maturation of the extracellular matrix, collagen and elastin maturation and stability, antioxidant defence mechanism, wool and hair pigmentation, activation of neuropeptides, and neurotransmitter synthesis are examples of

its ubiquitous nature (NRC, 2005; Aggett, 2012). Copper is also required for erythrocytes formation via ceruloplasmin (Cp) and hephaestin (HEP); Cp serve in transporting Fe from storage organs to the site of erythropoiesis, while HEP exports Fe from the intestine to storage sites in the body (Prohaska, 2012). In addition, Cu stimulates structural integrity of the bone collagen and elastin formation of the vascular system through the enzyme lysyl oxidase (Suttle, 1986). Myelination of the neurons and spinal cord require Cu as it is a structural unit of the cytochrome oxidase enzyme; probably the myelination process is impeded during development which leads to myelin aplasia, associated with degeneration of the motor neurons of the brain and spinal cord in Cu deficient new-born lambs causing a disease named swayback (Suttle, 2010).

Table 1.5. Copper containing enzymes and their functions in mammals (Prohaska, 2012)

Enzyme	Function
Ceruloplasmin	Fe^{2+} oxidation (Fe^{2+} to Fe^{3+} oxidation)
Cytochrome c oxidase	Electron transport during aerobic respiration in cells, thus for energy generation in all tissues
Dopamine-β-monooxygenase (secretory enzyme)	Norepinephrine production from dopamine
Hephaestin	Fe^{2+} oxidation (Fe^{2+} to Fe^{3+} oxidation)
Lysyl oxidase	Crosslinking of collagen and elastin
Peptidylglycine α-amidating monooxygenase	Peptide C-terminal α-amidation of peptide hormones for release
Cu/Zn superoxide dismutase	Oxidant defence: superoxide radical detoxification
Tyrosinase	Melanin synthesis, DOPA quinone formation
Zyklopen	Fe^{2+} oxidation
Diamine oxidase	Oxidative deamination of diamines and their derivates
Sulfhydryl oxidase	Keratin cross-linking
Blood clotting factors V, VIII	Blood coagulation system

1.5. Clinical signs of copper deficiency

Copper deficiencies in sheep can be classified into primary, and secondary deficiency (NRC, 2005). In primary Cu deficiency, there is a lack in dietary Cu intake, while in secondary Cu deficiency, dietary Cu is adequate, but the deficiency develops due to the impairment of Cu absorption and/or abnormal utilization of ingested Cu (Gould and Kendall, 2011). Presence of high Mo, S, and Fe concentration in the diet of ruminants are the main causes of secondary Cu deficiency (Wikse *et al*., 1992; Radostits *et al*., 2006). Copper antagonistic interactions with other minerals is often attributed to inhibition of intestinal absorption, or to the antagonism that takes place at the cellular level (Henry and

Miles, 2000). Copper interactions with other minerals will be further discussed in section 1.9.1.

Suttle (2010) divided Cu deficiency of ruminants into four phases named: depletion, deficiency, dysfunction, and clinical disease (Figure 1.1). During the depletion phase, there is a reduction in the tissue (mainly liver) and fluid Cu levels. However, plasma Cu concentration may not change during this phase (Gengelbach *et al.*, 1997). When Cu deficiency continues, liver Cu reserves will become exhausted and cannot supply enough Cu to maintain blood Cu concentration resulting in marginal deficiency; liver Cu concentration of 6.34 - 19.02 mg/kg DM or plasma Cu concentration of 3 - 9 µmol/l was determined to identify marginal Cu deficiency in weaned or adult sheep, goats and cattle (Suttle, 2005). Dysfunction, during this phase the activity of Cu containing enzymes and hormones may decline, and there is no determined reliable measure of Cu dysfunction in animals yet (Suttle, 2005). However, dysfunction of Cu metabolism can occur with normal plasma Cu and liver Cu (Phillippo *et al.*, 1982). Finally, the disorder phase develops which is characterised by changes in cellular functions and is manifested as clinical signs of the disease and reduction in animal performance (Cerone *et al*., 2000; Radostits *et al*., 2006).

A great deal of Cu responsive disorders such as losing hair or wool colour and swayback in sheep and goats have been associated with a low Cu status of animal and the clinical term ‘hypocuprosis’ is usually uses to describe Cu deficiency in ruminants (Suttle, 2010). Susceptibility to Cu deficiency depends on the species of animal and on the stage of development at the time when Cu deprivation takes place (McDowell, 2003; Suttle, 2012). The most recognizable clinical sign of Cu deficiency observed in the neonatal animals is swayback (hind limb ataxia) which is due to demyelination of the spinal cord, that occurs especially in sheep (Wiener, 1971; Suttle, 1988; Faye *et al*., 1991) and goats (Wouda *et al.*, 1986).

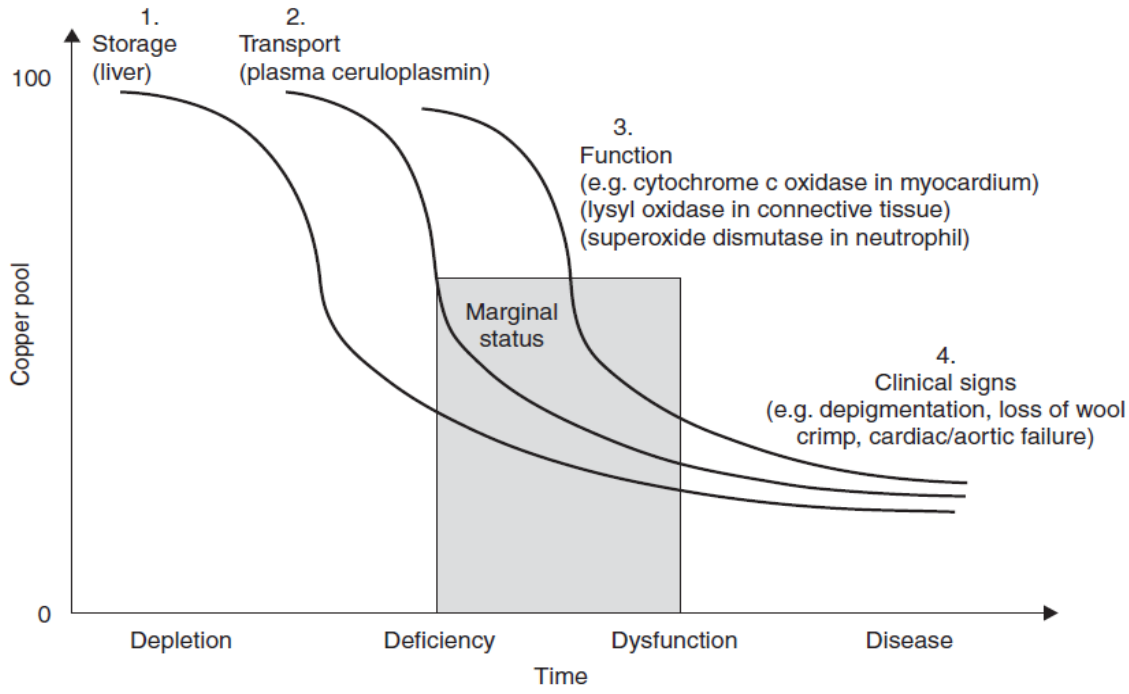

Figure 1.1. Sequence of the biochemical changes brings to the appearance of clinical signs of copper deficiency (Suttle, 2010).

The ewes that produce affected lambs with swayback have also been found to have lower blood Cu concentrations compared with ewes that produced normal lambs (Wiener *et al*., 1969; Wiener, 1971). Howell and Davison (1959) and Mills and Williams (1962) found a lower activity of cytochrome C oxidase in the degenerating nervous tissue that coincided with lowered brain Cu concentrations (<3.0 mg/kg DM) and the neuronal chromatolytic changes characteristic of enzootic ataxia.

The brain and central nervous system (CNS) are vulnerable to Cu deficiency (Harris, 1997). Typically, neuropathologies resulting from Cu deprivation were first observed in new-born lambs. The symptoms were incoordination of the hindquarters, with ataxia, tremors, and a swaying gait (Morgan, 1980; Pugh and Baird, 2012). The ataxia, tremors, clonic seizure, hypomyelination, or demyelination appear to be linked to reduced levels of sphingolipids and a reduction in the steady-state concentrations of norepinephrine in the CNS (O'Dell and Prohaska, 1983). The low norepinephrine levels possibly links to lower concentration of dopamine-*β*-monooxygenase, a Cu dependent enzyme. In the swayback lamb (O'Dell *et al.*, 1976) and in the Cu deficient rat (Morgan and O'dell, 1977), necrosis occurs in certain areas of the brain, particularly the corpus striatum. In these species, Cu deficiency results in reduced dopamine in addition to norepinephrine levels (Harris, 1997).

The other common signs of Cu deficiency in animals are anaemia, growth impairment, bone disorders, depigmentation of hair and wool, myocardium fibrosis, impairment of immune function, and diarrhoea (McDowell, 2003; Leeson, 2009). Anaemia usually

develops only when Cu deficiency is severe or sustained (NRC, 2005). Depressed fertility associated with delayed oestrus seems to occur in cows grazing Cu deficient pastures but this relationship is inconsistent (Gooneratne *et al.*, 1989; Suttle, 2010). Apparently, reproduction impairment is more related to secondary Cu deficiency than to the primary Cu deficiency (Phillippo *et al.,* 1982).

Severe Cu deficiency resulted in the loss of the integrity and elasticity of connective tissue in mammals which increase the fragility of the blood vessel walls, abnormal elastin, vascular lesions, and a greater possibility of aneurysms have also been reported (Allen and Klevay, 1978; McDowell, 2003; Suttle, 2010). Impairment of the cross linkage of collagen and elastin fibres was also observed in Cu deficient cattle (Bonham *et al*., 2002). Dysfunction of connective tissues leads to severe tendon and skeletal abnormality (Thompson *et al*., 1994; Werman and David, 1996). Increased rates of mortality have also been reported in sheep raised on low Cu status pastures and was linked to the increases in lamb infection (Suttle and Jones, 1986). Similar observations have also been found in Cu deficient mice (Jones and Suttle, 1983), and rats (Newberne *et al.*, 1968). Antimicrobial activity of neutrophil cells and their phagocytic ability declined in calves given a Cu deficient diet (induced by Fe and Mo supplementation) compared with those given a Cu adequate diet (Jones and Suttle, 1981; Boyne and Arthur, 1986). Gengelbach *et al*. (1997) observed lower neutrophil bactericidal and SOD activity of calves fed a marginally Cu deficient diet (6 - 7 mg/kg DM) supplemented with 5 mg Mo/kg DM compared with calves given 10 mg supplemental Cu/kg DM.

1.6. Copper toxicity

Copper requirements of ruminants are governed by species, source, mineral interactions, and physiological status of animal (McDowell, 2003; NRC, 2005). The recommended level of Cu for one species may cause toxicity in another. In addition, sheep accumulate Cu in their livers more readily than other farm animals species that make them prone to Cu toxicity (NRC, 2005). Copper toxicity may take place by a large single dose of Cu (acute) or it occurs as a result of the repeatedly exposure to Cu concentrations that exceed its requirements (chronic) (NRC, 2005). Even with moderate dietary Cu intake, the liver tends to accumulate high amounts of Cu that could lead to liver failure and possibly death especially when low concentrations of Cu antagonists is present in the diet such as a low Mo supply (Ishmael *et al.*, 1971; Pope, 1971). Variable incidence of Cu toxicities could partly attributed to the differences in the efficiency of Cu absorption and excretion (Bremner, 1998).

Pre-ruminant calves are more susceptible to Cu toxicity due to the high efficiency of Cu absorption (about 75%), but after weaning Cu absorption decrease as a result of the

action of rumen microorganisms that reduce dietary S to sulphide that reduces Cu availability (Buckley, 2000; Gould and Kendall, 2011). Copper absorption of the milk-fed lamb is high, about 70 – 85%, but in weaned lambs is very low, <0.1 - 1.0%, (Suttle, 2010). On the other hand, pigs are tolerant to higher levels of dietary Cu with intakes of 250 mg Cu/kg DM stimulating growth (Bremner, 1998). The European Union (EU), under Commission Regulation 1334/2003, has enforced lower (non-pharmacological) levels of Cu and Zn in pig diets, which applied from 26 January 2004 to reduce environment pollution (Ec, 2003). European food safety authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) recommends that Cu content in complete feed for piglets should not exceed 25 mg/kg (down from 170 mg/kg) on 9 August 2016. EFSA has proposed modifications to the maximum levels of Cu in feed for some animal groups including piglets, cattle and dairy cows, and an increase in feed for goats. The proposed levels would reduce the amount of Cu released into the environment. The high susceptibility of sheep to chronic Cu toxicity is due to their poor ability to control liver Cu balance, as biliary Cu excretion is not developed enough to control liver Cu accumulation to avoid toxicity, which makes sheep very vulnerable to Cu overloaded (Lockhart and Mercer, 2000).

In sheep, Cu toxicity consists of two phases. In the first phase (pre-haemolytic phase), no obvious signs of Cu toxicity can be observed and animal performance is not usually affected until shortly before the haemolytic phase; blood Cu concentration remain unchanged or slightly increased but elevation in the concentrations of liver specific enzymes in plasma have been reported (Bremner, 1998). This change reflects the gradual but extensive increase in hepatic Cu, and concentrations increasing to over 1000 mg/kg DM. Sheep, goat, and cows with liver Cu concentration of 400 – 1000 mg/kg DM are at risk of marginal Cu toxicity (Suttle, 2005). However, Animal and Plant Health Agency considered that animals with liver Cu values about 500 mg/kg DM are at risk of marginal Cu toxicity. This leads to necrosis of the parenchymal cells and to swollen Kupffer cells (Ishmael *et al.*, 1971). In the second phase of Cu toxicity (haemolytic phase), the disease happens very suddenly and it is usually referred to as a “haemolytic crisis”. The main clinical signs include jaundice, anorexia, excessive thirst, and haemoglobinuria (Ishmael *et al.*, 1971; NRC, 2005). Animals generally die during a few days as a result of the release of stored hepatic Cu and a massive increase in blood Cu concentrations, even though some animals can survive if they get quick treatment (Bremner, 1998). Supplementation of 0.1 g ammonium molybdate plus 1 g sodium sulphate/sheep/day was found to be very effective in a rapidly decreasing mortality in rams due to Cu toxicity (Hidiroglou *et al.*, 1984). A highly significant increase in the faecal Cu excretion was evident and the increase persisted throughout the monitoring period of rams. However, there was no significant effect of Mo and S supplementation on urinary excretion of Cu in

rams (Hidiroglou *et al*., 1984). Similarly, oral penicillamine treatment (50 mg/kg LW/d) induced cupruresis, excretion of excessive Cu in the urine, but did not affect faecal Cu excretion in the same animal group. Thiomolybdate injection (ammonium TTM) has prevented excessive liver Cu storage in adult Merino sheep receiving high Cu doses and reduced liver Cu concentration in sheep that were not given additional Cu (Gooneratne *et al.*, 1981). To investigate the systemic effects of TTM on Cu and Mo distribution and retention of sheep of different breeds (Cambridge and North Ronaldsay), and different Cu status (low, 3.4 mg/kg DM and high 150 mg/kg DM), Haywood *et al*. (1998) found that Mo was accumulated significantly in all investigated organs (brain, liver, kidney, heart, skeletal muscle, pituitary, adrenals, testes and ovaries) in all TTM treated groups and was retained after cessation of treatment, except in liver, kidney and skeletal muscle. Copper increased and was retained in the cerebellum and medulla oblongata in the high-Cu fed Cambridge groups treated with TTM. Concentrations of brain Cu *vs*. Mo showed a strong positive correlation (r= 0.7) in high-Cu fed Ronaldsay lambs after TTM treatment by 7 months. The researchers has also concluded that TTM is not excreted completely but Mo has been found to be widely distributed and retained in several organs including brain and pituitary. Besides, TTM may redistribute some displaced excess liver Cu (Cu-TTM) to the brain.

In the UK, the number of chronic Cu poisoning cases reported increased gradually to about 100 per annum between year 1970 and 1990, this was partially due to the growing popularity of susceptible sheep breeds such as the Texel (Suttle, 2010). North Ronaldsay sheep breed can succumb to Cu toxicity while grazing on normal pastures (Wiener *et al.*, 1978); North Ronaldsay is a primitive sheep breed which occupies an ecological position on the Cu-impoverished foreshore of North Ronaldsay island, and have adapted to Cu absorption and storage (MacLachlan and Johnston, 1982). When moved to the mainland, North Ronaldsay sheep are in danger of succumbing to Cu toxicity (Wiener *et al.*, 1977). North Ronaldsay and Cambridge breeds were compared in respect of the pathological changes and expression of proteins important in Cu metabolism in the liver as a result of excessive dietary Cu intake (15 and 155 mg Cu/kg DM, respectively), Haywood *et al.* (2005) observed an acute mitochondrial damage and activation of hepatic stellate cell, also known as perisinusoidal cells or Ito cells found in the perisinusoidal space of the liver, activation with collagen synthesis observed in North Ronaldsay but not in Cambridge sheep in response to the moderate Cu overload. Changes such as mitochondrial degradation happened either as swelling degeneration and rupture with subsequent autophagic degradation or mitochondrial matrical condensation (pyknosis). The continued exposure of animals to Cu produced mitochondrial hyperplasia and hypertrophy, and other nuclear damage beside necrosis in North Ronaldsay sheep. Cytosolic isocitrate dehydrogenase (IDH), an enzyme responsive to oxidative stress, was produced in the Cambridge sheep liver given Cu supplemental diets but was undetectable in the control

sheep. In contrast, IDH was found at similar levels in both control and Cu supplemented North Ronaldsay sheep, indicating a lower threshold response, and an enhanced susceptibility, to oxidative stress (Haywood *et al*., 2005).

In some parts of Australia and elsewhere, normal intakes of Cu with low Mo (0.1-0.2 mg/kg DM) or the consumption of specific plants such as Heliotropium europaeum, that contain hepatotoxic alkaloids (heliotrine and lasiocarpine), may led to 'toxaemic jaundice' or 'yellows', which both cause Cu toxicity in ruminants (Howell *et al*., 1991). Chronic Cu toxicity more frequently occurs in housed lambs, milk sheep and pedigree rams recieving high concentrate diets; it is also found in sheep housed on slatted floors compared with those bedded on straw (Suttle, 2010). Feeding high Cu contenting palm-cake (contained 25 - 40 mg Cu/kg) caused Cu toxicity in sheep (Chooi *et al*., 1988). Reports have detailed Cu toxicities in Texel sheep which had received neither supplementary feeding nor parenteral Cu administration (Brooks, 1998; MacPherson *et al*., 1997).

During the haemolytic phase of Cu toxicity, a significant decreases in blood haemoglobin (Hb) and glutathione concentrations can be observed within a few days, with a temporary elevation in blood methemoglobin concentration (Bremner, 1998). Sheep usually die within a few days although a few can survive. The onset of these signs is associated with liberation of hepatic Cu and an enormous increase in blood Cu concentrations. The activity of the liver-specific enzymes increase more in the plasma, consistent with extensive liver degeneration and with the occurrence of focal necrosis, inflammatory cells, bile plugs, and large periodic acid–Schiff (PAS)-positive Kupffer cells in liver samples (Bremner, 1979). Considerable kidney damage was also observed, with necrosis and loss of mitochondrial enzyme activity from the proximal convoluted tubules. Kidney copper concentrations increase dramatically at this time, probably because of enhanced tubular reabsorption of circulating Cu (Bremner, 1998).

1.7. Copper Metabolism

Monogastrics have developed effective mechanisms to control and regulate uptake, distribution, storage, and excretion of Cu (Figure 1.2) to maintain Cu balance (Nordberg *et al*., 2014). Differences in Cu metabolism have been reported between ruminant and non-ruminant species (NRC, 2005), and also between ruminants such as sheep and deer (Freudenberger *et al*., 1987), cows and sheep (ARC, 1980), and between goats and moose (Frank *et al*., 2000), or between individuals within the breed (Wiener and Woolliams, 1983; Littledike *et al*., 1995; Du *et al*., 1996).

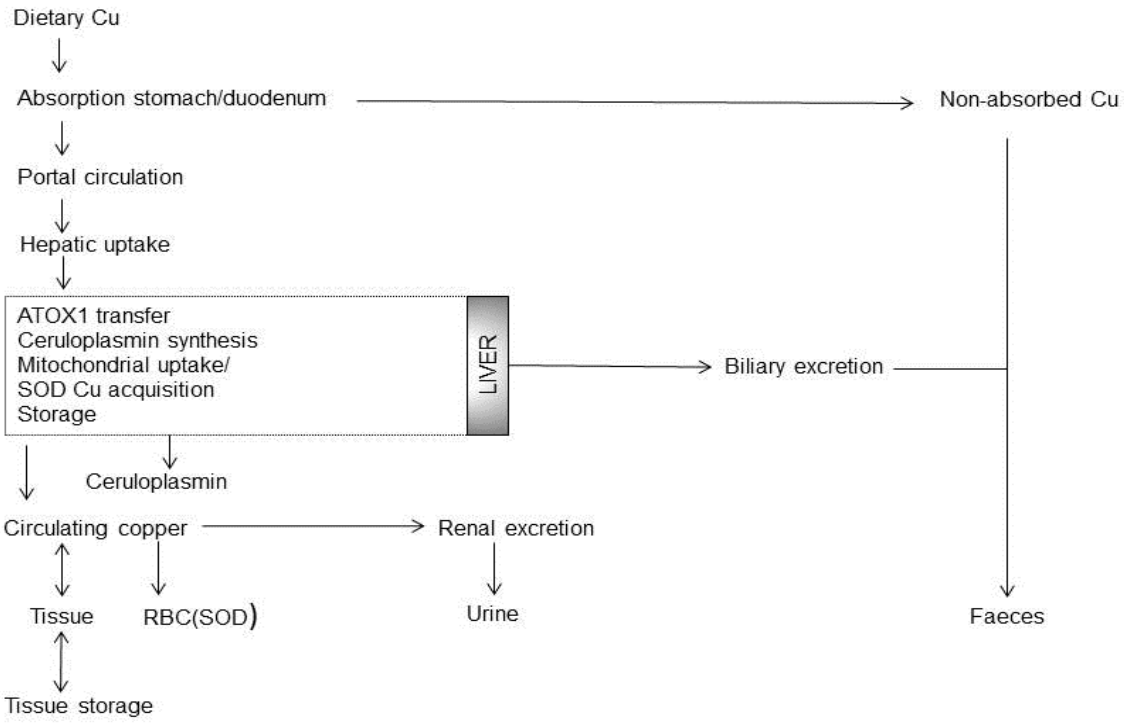

Figure 1.2. Copper metabolism (Adapted from: Schilsky and Thiele, 2009)

1.7.1. Absorption

Limited information is available about the site of Cu absorption in ruminants (Mohammed *et al.*, 2016). It has been reported previously that Cu may be absorbed from the small and large intestines in sheep (Miller *et al.*, 1988) while earlier studies suggested that the major site of Cu absorption in sheep is the large intestine (Hoover, 1978). The main site of Cu absorption in man (Bush *et al.*, 1955) and rats (van Campen and Mitchell, 1965) is reported to be the stomach, and in chicks is the duodenum (Starcher, 1969). In recent reports in mammals, it has been found that Cu is predominantly absorbed in the upper section of the small intestine, mainly the duodenum, (McDowell, 2003b; NRC, 2005; Lutsenko *et al.*, 2007; Vonk *et al.*, 2008; Kodama *et al.*, 2012), and perhaps in the stomach (Linder, 1991a; DiDonato and Sarkar, 1997).

Specific Cu transporters and chaperones are in place to control Cu metabolism in mammals at the cellular level. Dietary Cu from the intestinal lumen is taken up by two important protein transporters: the high affinity Cu transporter 1 (CTR1), after cupric Cu is reduced to cuprous Cu (Cu^{+2} to Cu^{+1}) by an intestinal reductase (Prohaska and Gybina, 2004). The second transporter protein is divalent metal transport DMT present in the intestinal mucosa (Hansen *et al.*, 2008; Prohaska, 2012)

Pre-ruminant animals have the ability to absorb Cu as efficiently as monogastric species and more efficiently than mature ruminants (McDowell, 2003; NRC, 2005). The reason

behind the low Cu absorption in the mature ruminants is due to the antagonistic interaction with other minerals in the rumen (see section 1.9). Copper absorption varies greatly between animals depending on species and dietary factors (Buckley, 2000). In ruminants, Cu absorption ranges from <0.01 to 1.0% or lower (Suttle, 2010), but it ranges from 12 to 55% in humans (Buckley, 2000). Weaned ruminants have a poor ability to absorb dietary Cu due to the presence of microorganisms in the functional rumen that produce sulphides from the digestion of organic and inorganic sulphur, with rumen protozoa being a significant generator of sulphide, that has been reported to complex with Cu and reduce its availability for absorption (Spears, 2003; Suttle, 1991). A lot of the Cu released through the ruminal digestion of feedstuffs is reported to be precipitated as copper sulphide (CuS) (Suttle *et al*., 1984; Gould and Kendall, 2011). High dietary Mo and S concentrations results in thiomolybdate (TM) formation in the rumen which complex Cu and further reduce its absorbability (Suttle, 1991; McDowell, 2003; Gould and Kendall, 2011) (see Section 1.9.1).

Absorption, excretion, and Cu incorporation into metalloenzymes does not occur through passive diffusion or at any degree of spontaneity, as free or unbound Cu is highly toxic to the cell (Fry, 2011). Previously suggestions that Cu is absorbed into the body through simple diffusion (Bronner and Yost, 1985) have now been dismissed due to the discovery of the specific transport proteins (Prohaska, 2012).

Metallothionein (MT), a low molecular weight protein containing Zn and Cu, that is present in the mucosal membrane of the small intestine, sequesters Cu in the intestine providing protection from excessive Cu transfer to blood, while in the liver it serves as a primary Cu storage protein. Hence, MT can function in Cu homeostasis, storage, transport, and detoxification (Evering *et al.*, 1991). Upon entering the enterocyte, Cu is bound to one of several Cu chaperones; Cu can be delivered to cytochrome oxidase (CCO) in the mitochondria via COX17 where Cu is delivered to a series of other CCO assembly proteins and subsequently CCO subunits (Figure 1.3). Antioxidant 1 (ATOX1) chaperones Cu down the secretory pathway in the trans-Golgi network where Cu is delivered to one of two Cu dependent Cu-ATPases, ATP7A in the intestine and ATP7B in the liver (Hamza *et al.,* 2003; Hamza *et al*., 1999). These ATPases are dual function Cu transporters. ATP7A is responsible for basolateral Cu export from the enterocyte and incorporation of Cu into cuproenzymes (i.e. lysyl oxidase, tyrosinase). While, in the liver (hepatocyte), ATP7B interacts with MURR1 domain 1 (COMMD1) to excrete Cu via bile, the major route of Cu excretion in mammals. ATP7B is also responsible for incorporating Cu into Cp.

Zinc, but not Cu, induces MT synthesis in cells of the intestinal mucosa, through increases in the transcription of MT mRNA (Menard *et al*., 1981; Harris, 1997). Metallothionein (MT) present in the mucosal membrane of the intestine binds dietary Cu and that may be lost in

faeces during the natural turnover of intestinal cells (Linder, 2002). n rats, Fischer *et al.* (1981) measured a significantly lower Cu concentration in the intestinal mucosal cells of rats fed no supplemental Zn compared with those given Zn supplements (7.5, 15, 30, 60,120 and 240 mg/kg DM), but Cu concentration increased in the rats fed Zn supplements in a dose response order (211.2, 236.0, 265.6, 293.7, 296.0 and 304.9 mg Cu/kg, respectively). These findings confirm that supplemental Zn can bind dietary Cu in the mucosal cells and prevented from absorption.

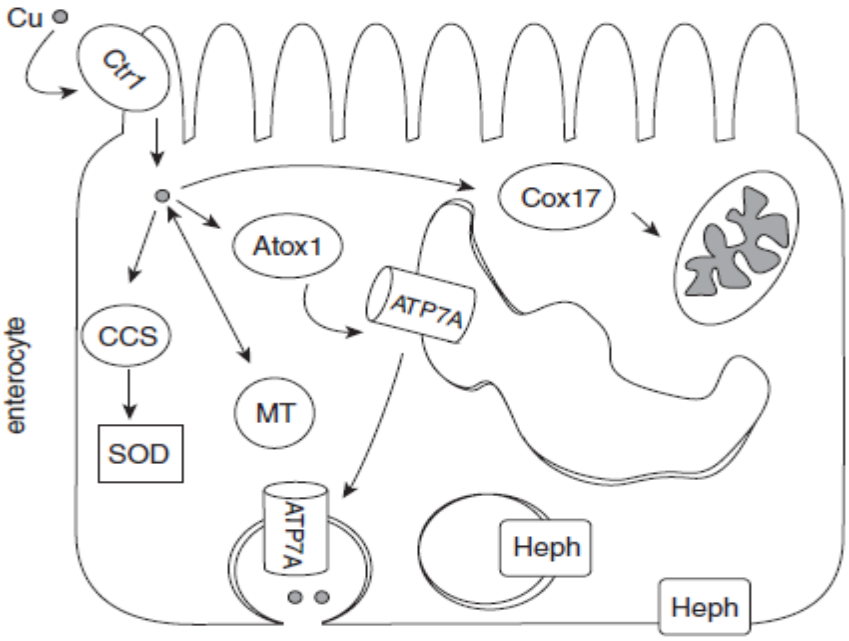

Figure 1.3 Cellular transport and excretion (enterocyte) of copper into the blood stream (Prohaska, 2012).

Metallothionein was shown to have a higher affinity to bind with Cu than Zn in the intestine besides high dietary Zn induces transcription and translation of MT and subsequently leads to reduced Cu absorption (Harris, 1997) Therefore the mucosal MT might be a "stumbling block" to Cu absorption from the small intestines and prevents Cu toxicity (Linder, 1991; Linder, 2002).

1.7.2. Distribution and storage

Copper enters the portal circulation from enterocytes via the ATP7A transporter protein, then it binds to transporter proteins such as albumin and transcuprein, and also the amino acid histidine which play an important role in Cu transport to the liver (Weiss and Linder, 1985; Harris, 1991; Linder, 2002; Vonk *et al.*, 2008; Prohaska, 2012). Albumin is recognised as the main transporter protein responsible for Cu transport from the intestine

to liver (Linder, 2002). Particular receptors are present on the surface of liver and kidney cells that are sensitive to uptake Cu associated with albumin and transcuprein (Linder, 2002; Prohaska, 2012).

Copper enters hepatocytes via plasma membrane protein Ctr1 (Peña *et al.*, 1999). In the hepatocyte, the newly arrived Cu requires several chaperones to incorporate cuproenzymes, store, or excrete into bile (Figure 1.4). Liver has the capacity to store approximately 20% of the body's Cu supply in adult human. While muscle tissue (40%), brain (20%), connective tissue (8%), blood (8%) and kidneys (8%) can store and utilize Cu (Dameron and Howe, 1998). In sheep, liver contains about half of the body Cu (Grace and Clark, 1991). Atox1 is a chaperone that delivers Cu to the secretory pathway (Klomp *et al.*, 1997; Llanos and Mercer, 2002; Aggett, 2012).

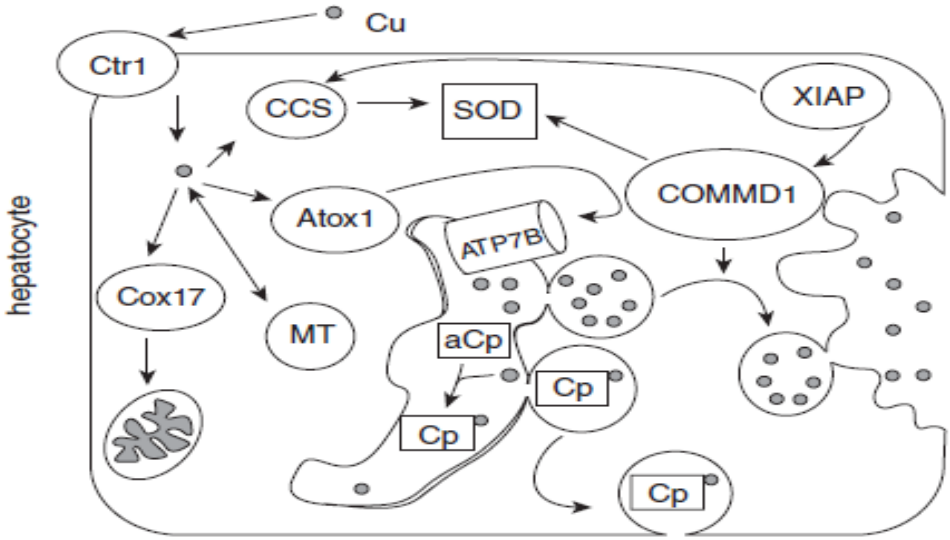

Figure 1.4. Cellular copper transport and excretion (hepatocyte) (Prohaska, 2012).

Ctr1 deliver Cu to ATOX1 which in turn interacts with ATP7B in the liver or ATP7A in other tissues. ATP7B guides Cu to biliary excretion or to the Cp for excretion into plasma. While ATP7A directs Cu to dopamine β-monooxgenase, peptidylglycine α-amidating monooxygenase, lysyl oxidase and tyrosinase, depending on the cell type (Prohaska and Gybina, 2004). Another important chaperone is CCS which is required to deliver Cu to the SOD enzyme (Culotta *et al.,* 1997). Cox17 is a chaperone that delivers Cu to the cytochrome c oxidase through the Cox11, Sco1 and Sco2 chaperones (Glerum *et al.*, 1996). In the liver metallothionein (MT) is a potential chaperone and may be important in the intracellular transfer and storage of Cu (Coyle *et al.*, 2002). The proteins involved in Cu homeostasis of mammals are present in Table 1.6.

Table 1.6. Proteins involved in mammalian copper homeostasis (Adapted from Prohaska, 2012)

Protein	Function
Albumin	Plasma copper transport
CTR1	Copper uptake in intestine and liver
CTR2	Copper uptake in intestine and liver
DMT1	Copper uptake in intestine
Copper chaperone for superoxide dismutase (CCS)	Intracellular copper trafficking
ATOX1	Intracellular copper trafficking
Cox17	Intracellular copper trafficking
Glutathione (GSH)	Intracellular copper trafficking, storage and detoxification
Metallothionein (MT)	storage and detoxification
ATP7A	Copper efflux (intestine cells)
ATP7B	Copper efflux, Cp metalation (liver cells)
COMMD1	Biliary Cu excretion, SOD1 assembly

1.7.3. Copper excretion

The liver is the main storage and distribution organ of Cu in animals and subsequently is responsible for Cu homeostasis (Dameron and Howe, 1998). Uptake of Cu across the hepatic membrane occurs via a carrier mediated mechanism and is not dependent on cell energy or coupled to a sodium (Na) gradient (McArdle *et al*., 1999). In humans, biliary Cu excretion takes place in association with the biliary glutathione excretion at the last step of Cu excretion from hepatocyte into bile (Dameron and Howe, 1998). Hamza and Gitlin (2004) reported that under normal conditions the amount of Cu excreted into bile is proportional to the size of liver Cu pool. In the liver Cu is incorporated into Cp and other Cu dependent enzymes such as Cu-Zn superoxide dismutase (SOD). Ceruloplasmin is the carrier for the tissue specific export of Cu from the liver to the target organs (McDowell, 2003). Approximately 90% of Cu in plasma is bound to Cp with the rest being bound mainly to albumin, transcuprein and ligands (McArdle *et al*., 1999). Copper in the liver can be mobilized to extrahepatic tissues when the body is in negative Cu balance (Dameron and Howe, 1998). In all species, large amounts of ingested Cu appears to be excreted through the faeces (McDowell, 2003).

Copper transporting P-type ATPase, ATP7B is expressed within the secretory pathway of hepatocytes (Hamza and Gitlin, 2004). It is important in Cp secretion from hepatocytes

that is important in Fe oxidation and efflux from the liver and other storage organs. ATP7B and Cu metabolism COMMD1 are responsible for the biliary Cu excretion (Fry, 2011).

Biliary Cu excretion is consider an active route of endogenous Cu excretion from the liver into intestine (Gooneratne *et al.*, 1989), and is responsible for Cu homeostasis in mammalians (Evans, 1973; Underwood, 1977). Eighty percent of excreted body Cu takes place via biliary excretion in humans (Winge and Mehra, 1990). Nevertheless, biliary Cu excretion mechanisms is less active to maintain Cu homeostasis and regulating hepatic Cu concentration in ruminants (Bremner, 1998) compared with monogastric species (NRC, 2005). In sheep, increases in dietary Cu intake did not significantly increased biliary Cu excretion in accordance to the intake levels (Saylor and Leach, 1980; NRC, 2005). However, in sheep biliary Cu excretion increases linearly with an increase in the liver Cu concentration above 79.43 mg/kg DM (Grace *et al.*, 1998). Yu *et al*. (1994) observed a significant positive correlation between biliary Cu excretion and the liver Cu concentration of rats. Biliary Cu excretion was reported to increase with increasing Cu consumption in rats, cattle and chickens, but not in sheep (Buckley, 2000). Urinary Cu excretion in sheep is very low (0.047 ± 0.018 mg/d) (Suttle, 1974c) and is reported to be constant and not affected by dietary Cu intake or liver Cu concentration, making urinary Cu excretion a non-significant route of excretion. However, it has been shown to increase with increases in dietary Mo ingestion (Smith *et al.*, 1968; Pott *et al.*, 1999). The remainder of the Cu is excreted in smaller quantities through milk, and small amount is excreted in perspiration (McDowell, 2003; Suttle, 2010).

1.8. Proteins involved in copper acquisition, distribution, and utilization

Numerous transport and chaperone proteins exist to regulate Cu acquisition, intracellular distribution, and utilization in animals. They are essential to prevent both deficiency and toxicity of Cu (Kim *et al*., 2008; van den Berghe and Klomp, 2010). The Cu transporter proteins includes:

1.8.1. Copper transporter 1 (CTR1)

Copper transporter 1 (CTR1) is an essential integral membrane protein of the CTR protein family, a family of transporters that are highly conserved in eukaryotes, that deliver Cu^{+1} to the intracellular Cu chaperone proteins (van den Berghe and Klomp, 2010). CTR1 is located on the apical surface of the enterocyte (Nose *et al*., 2010). The experimental evidence strongly supports that Ctr1 transports Cu^{+1} across membranes, with Cu^{+2} likely converted to Cu^{+} by metalloreductases present on the cell-surface prior to be transport by Ctr1 (Rees and Thiele, 2007). Lee *et al.* (2002b) reported that CTR1 is specific for Cu^{+1} but Zn, Fe, and cadmium (Cd) are the possible inhibitors or are of low affinity substrates to the CTR1 uptake when present at high concentrations.

Lee *et al*. (2001) reported deletion of the CTR1 gene resulted in compromised growth and development with mice dying in utero at mid-gestation. Nose *et al*., (2006) reported that intestinal epithelial cell-specific knockout CTR1 mice suffer greatly from Cu deficiency. Knockout hepatocyte specific CTR1 mice showed a reduction in growth, liver Cu, biliary Cu excretion, and a decline in the activity of the Cu-dependent enzymes including SOD1, CCO, and Cp (Kim *et al*., 2009). Kim *et al*. (2009) proposed that other mechanisms involved in Cu acquisition possibly compensated for the 90% reduction in CTR1 protein expression, since Cu deficiency was minor and not as severe as that found with intestinal CTR1 deletion. Brain tissue has a high Cu demand also and the work by Gybina and Prohaska (2006) has revealed that Cu deficiency raises CTR1 protein in the rat choroid plexus. Transcript levels of CTR1 have also been recognised in the rats placenta, nevertheless they were not affected by Cu deficiency (Andersen *et al.*, 2007). Studies indicated that intestinal and cardiac expression of CTR1 protein was greater in Cu deficient rats (Nose *et al.*, 2010).

1.8.2. Antioxidant 1 (ATOX1)

One of the Cu chaperones where CTR1 deliver Cu^{+1} is ATOX1, which is a vital secretory Cu chaperone (Prohaska and Gybina, 2004). ATOX1 knockout studies, confirmed the biochemical importance of it in mammals. ATOX1 deletion dramatically reduces growth and survivability; with about half of mouse pups dying by mid-gestation (Hamza *et al*., 2001). Pups that survived were characterised by a common signs of clinical Cu deprivation, such as loss of pigmentation, skin slouching and growth and development retardation, with these characteristics being observed toward maturation after weaning. Deletion of ATOX1 reduced tyrosinase activity, but growth and mortality between both groups of pups did not differ ($Atox1^{+/-}$ and $Atox1^{+/+}$) (Hamza *et al*., 2001). Hamza *et al*. (2003) found the importance of Cu delivery by ATOX1 to ATP7A. Similar results have been reported by Walker *et al*., (2002) regarding ATP7B, in which ATOX1 controlled the amount of the Cu ions delivered to ATP7B besides ATOX1 catalytic activity.

1.8.3. Copper-dependent ATPases

Copper dependent ATPases, ATP7A and ATP7B, are important in Cu homeostasis (La Fontaine *et al.*, 2010). These proteins are approximately 60% homologous and contain eight transmembrane domains with their amino and carboxy termini located in the cytosol. Both proteins are dual-functioning in that they are involved in synthesis of cuproenzymes and Cu efflux. There are two inherited diseases in human occurs due to mutation in ATP7A and ATP7B gene function named Menkes' disease and Wilson disease. ATP7A is the protein missing or mutated in humans with Menkes' disease that result in Cu deficiency. While ATP7B is the protein mutated in Wilson's disease and leads to hepatic Cu toxicity (Prohaska, 2012).

1.8.3.1. Copper-dependent alpha polypeptide ATPase (ATP7A)

Previous data has demonstrated that ATP7A localisation is dependent on Cu concentrations (Prohaska and Gybina, 2004). Lutsenko and Petris (2002) reported that when cells were exposed to high concentrations of Cu, ATP7A moved from the trans-Golgi network (TGN) to the plasma membrane to promote Cu efflux. Nyasae *et al*. (2007) examined ATP7A localization in polarized intestinal epithelia, rat enterocytes, and Caco-2 cells. Nyasae *et al*. (2007) have found that in all cell types, under conditions of Cu deprivation an abundance of ATP7A was localised to the TGN. However, when Cu concentrations were increased, ATP7A localised to the cell periphery. Nyasae *et al.* (2007) reported that ATP7A protein observed to be most abundant in the upper jejunum

and was lowest in the ileum with duodenal expression being intermediate. The authors suggested Cu absorption from the enterocyte for utilisation may occur from the upper jejunum given the abundance of ATP7A protein in this section of the intestine.

1.8.3.2. Copper-dependent beta polypeptide ATPase (ATP7B)

ATP7B is a Cu transporting ATPase that is involved in Cu trafficking and homeostasis in the liver (Linder, 2002). Unlike the other known P-type ATPases, it possesses six homologous metallic binding domains at the N-terminal end (Lutsenko *et al.* 2008). Several mutations in the gene coding for this protein transporter lead to Wilson disease, a hepatic disorder characterised by impaired excretion of Cu in the blood and bile, and accumulation of Cu in the liver, brain, kidney, and eye cornea (Lutsenko *et al.,* 2008). ATP7B is a Cu ATPase which present primarily in the liver (Schaefer and Hopkins, 1999).

Similar to ATP7A, trafficking of ATP7B also occurs in response to intracellular Cu concentrations (Lalioti *et al.,* 2016). The membrane protein responds to changes in intracellular Cu levels by cycling between the Golgi and apical region. Guo *et al*. (2005) were using polarised hepatic cells (WIF-B) and high-resolution confocal microscopy to map the path of endogenous and exogenous ATP7B using different Cu conditions. In the Cu-depleted cells, the authors found that ATP7B resided in a post-TGN compartment, but in the Cu-loaded cells, the ATP7B relocated to unique vesicles close to the apical plasma membrane as well as the membrane itself. The authors indicated that the bile canaliculus is the primary site of ATP7B action to eliminate excess Cu^{+1} from the liver (Lalioti *et al.,* 2016). Hernandez *et al*. (2008) also reported that under conditions of low Cu exposure, ATP7B resided in the TGN.

1.8.4. Metallothionein

Metallothionein (MT) is a low molecular weight, cysteine-rich metal binding protein that has been characterised in numerous tissues, particularly the intestine, liver, and kidney and provides protection from Cu, Zn, and cadmium toxicity (Cousins, 1985). Metallothionein can also be found in the blood, urine and bile (Bremner *et al.*, 1986). In the intestine, high dietary Zn induces MT production that have a higher affinity to bound Cu than Zn and prevented from absorption, can be lost during the natural turnover of the enterocytes (NRC, 2005). On the other hand, MT in the liver serves as the primary storage protein for Cu (NRC, 2005). Additionally some reports in the literature have suggested that MT may also act as a Cu chaperone providing Cu to metalloproteins (Palmiter, 1998).

Different isoforms of MT such as MT-1, MT-2, MT-3 and MT-4 and other isoforms in various biological systems were discovered (Vašák, 2005). These isoforms are classified based on molecular weight, metal that bind, encoded genes, chromosomes, binding atoms, amino acids environment (Thirumoorthy et al., 2011). Generally, it is classified as major and minor groups, the major groups are MT-I and MT-II; these are the unique structure which is identical for the two major isoforms and binds 7 atoms of the divalent metals such as zinc and cadmium (Thirumoorthy *et al.*, 2011). The MT-3 and MT-4 are minor isoforms that are normally found in specialised cells (Thirumoorthy *et al.*, 2011). The majority of mammalian tissues have two isoforms of MT (MT1 and MT2) (Thirumoorthy *et al.*, 2011).

Recent work in cattle indicated that hepatic MT concentration was not correlated to liver Cu but was correlated to liver Zn level (López-Alonso *et al.,* 2005). However, when the authors considered the amount of Cu and Zn bound to MT, both minerals were correlated to MT content. Some breeds of sheep are quite susceptible to Cu toxicity, and work by Saylor *et al*. (1980) showed that Suffolk-Western Whiteface X Dorset have a limited ability to synthesize MT in the liver and intestine when high levels of dietary Cu are fed. In addition to providing protection against metal toxicity, MT can be induced by physiological stressors, chemical stressors, and compounds that promote oxidative stress (Sato and Bremner, 1993).

1.9. Copper interactions with other minerals in ruminants

Various minerals that are naturally found in animal diets can interfere with Cu absorption and metabolism. The minerals known to have antagonistic effects on Cu metabolism in animals include Mo, S, Ca, Zn, Fe, Mn, Co, Pb and Se (Underwood, 1981). Secondary Cu deficiency occurs as a result of the antagonistic interactions with the previously mentioned minerals when present in high concentrations in the animal's diet (Underwood, 1977). Secondary Cu deficiency is considered the principle cause of Cu deficiency in ruminants (Suttle, 1991). World reports associated with secondary Cu deficiency of animals, in which adequate concentration of Cu (6-16 mg/kg DM) were fed, were due to the presence of high concentration of Mo, S, and to other factors that reduce Cu utilisation in the body (McDowell *et al.*, 2001). The most important minerals that have been proven to have the greatest antagonistic effect on Cu metabolism of ruminants are S, Mo, Zn and Fe. Interactions with Ca and some transition metals are considered insignificant in the nutrition of farm animals (Graham, 1991).

1.9.1. Copper interaction with molybdenum and sulphur

Molybdenum is an essential trace element for all higher animals, it is a component of aldehyde oxidase, sulphite oxidase and xanthine oxidase (NRC, 2005). However, Mo requirements of animals are very low (NRC, 2005). In ruminants, Mo requirements have not been established but are estimated to be less than 2 mg/kg DM, this makes Mo deficiency rare in animals fed practical diets (Smith and Missen, 1982). High pasture Mo concentrations occur mainly in alkaline soils, and Mo toxicities is not normally observed in animals grazing pasture grown on acid (pH <6.5) and well-drained soils (McDowell, 2003). Suttle and Small (1993) reported that the average value of Mo in pasture was 1.1 mg/kg DM, (20 improved hill pasture sites) in Scotland, with 60 mg/kg DM being the highest concentration.

The first report that showed Cu metabolism in ruminants was affected by dietary Mo came in the early 1940's when a disease in cattle grazing pastures on Mo-rich soils in south west England was cured by spraying the pastures with $CuSO_4$ or via $CuSO_4$ supplementation of the drinking water (Ferguson *et al*., 1938; 1943). High dietary molybdenum causes depletion of Cu reserves of the animal, and develop Cu deficiency (Gooneratne *et al*., 1989).

Molybdenum generally is highly soluble in feeds and is unlikely to occur in undegradable forms that make it readily absorbed from the dietary sources (Suttle, 1991; McDowell, 2003). As Mo concentration in the diet increases in the presence of high S (from organic and inorganic source), thiomolybdates (TM) are produced in the rumen due to the reaction between Mo and sulphite due to the activity of microorganism (Gould and Kendall, 2011). TM may react with Cu to form physiologically unavailable complexes (Suttle, 1991). The Cu:Mo ratio in ruminant diets is important to determine Cu availability, where Cu deficiency may occur at ratios of 2:1 or less (Ward, 1978). Using a spectrophotometric investigation of the soluble TMs, when rumen microorganisms were added to the solution containing Mo and S, it has been shown that the products of the reaction between H_2S and molybdate ion in dilute neutral aqueous solutions are a series of salts of type $R^1{}_2Mo0^{4-}$ $n^S n$ where n=1 to 4, di-, tri-, and tetra-,TM molecules were formed (Aymonino *et al*., 1969). Dick *et al.* (1975) incubated a washed suspension of rumen micro-organisms in a solution containing unspecified amounts of molybdate and sulphate which led to the production of a mixture of TMs.

The first indication of the involvement of S in the Cu x Mo interaction came from studies in sheep when Dick (1953a; 1953b; 1953c) established that the S content of the diet was crucial in the interaction between Cu and Mo. Suttle (1974d) observed that both organic and inorganic S are equally important and reported that the total S content of the diet

should be considered in the interaction with Mo and Cu. Dick *et al*. (1975) observed that TM administration through a duodenal fistula resulted in the production of higher trichloroacetic acid-insoluble (TCA), the TCA-soluble Cu which is proposed by Tompsett (1934), is usually used to measure the available plasma Cu of ruminants, Cu fractions in plasma. However, infusion of Mo with or without S into the duodenum showed no effect on Cu in plasma. This was attributed to the absence of microorganism activity that reduces sulphur to sulphide.

Molybdenum has been found to have a slight effect on Cu status of sheep when Mo (from 0.5 to 4.5 mg/kg) was added to a semi-purified diet of sheep low in S (1.0 g/kg DM) (Suttle, 1974a). However, in sheep grazed summer herbage it was found that a small increase in the Mo concentration (from 0.7 to 1.2 mg/kg DM) significantly reduced intestinal Cu absorption (Suttle, 1981). This differences in the effect of dietary Mo on Cu status of sheep could be attributed to the presence of higher S concentration of the summer herbage compared to the semi purified diet (Suttle, 1983). Copper absorption was reduced by about 50% when the dietary Mo was increased from 2.5 to 4.5 mg/kg DM in sheep (Suttle, 1974a). Humphries *et al*. (1983) successfully induced the symptoms of Cu deficiency experimentally in calves fed a diet supplemented with 5 mg Mo/kg (basal diet contained 2.8 S g/kg DM) after 32 weeks.

Suttle (1974c) used a depletion-repletion technique to measure Cu absorption of sheep. In this experiment, hypocupraemic ewes (fed a purified diet containing 1.2 mg Cu/kg, 0.3 Mo/kg and 3-5 g S/kg on a DM basis) were compared with sheep given Cu (intravenously Cu infused) and the subsequent response in plasma was obtained to assess Cu intestinal absorption. In another study to investigate the effect of supplemental Mo-rich soils (10% of soil supplement) containing <2, 32, or 41 mg Mo/kg DM, on Cu status of hypocupraemic Scottish Blackface ewes, Suttle *et al*. (1975) found that the plasma Cu concentration reduced markedly in the groups given a diet supplemented with soils, but steadily increased in those given the soil-free diet. All soil supplemental diets reduced dietary Cu availability by over 50%, confirming that Mo from the soil is highly available for absorption. Bremner *et al*. (1987) found a significant reduction in the plasma Cu concentration in calves fed 4.12 mg Mo /kg DM) compared with those given no Mo supplements (7.87vs. 11.02 µmol/l, respectively). Phillippo *et al*. (1987a) and Gengelbach *et al.* (1994) also found a similar effect in cattle given a diet supplemented with 5 mg Mo/kg DM.

Woolliams *et al.*, (1983a) found a significant linear reduction in the hepatic and plasma Cu concentration of sheep fed a diet high in Mo (4.5 mg/kg DM, supplemental Mo) and low in Cu (4 mg/kg DM); liver Cu concentration decreased by 0.008 mg/d and plasma Cu concentration by 0.0016 µmol/l/d. Suttle (1974d) also found decreases in the plasma Cu concentration of sheep (by 39-56%) when dietary S concentration was increased from 1.0

to 3.0 or 4.0 g/kg DM; the dietary Cu availability was estimated to decrease from 6.2% to 4.1%.

The independent S effect was related to the production of an insoluble copper-sulphide (CuS) complex in the rumen as a result of sulphide produced by the microbial breakdown of dietary S compounds (Anderson, 1956). The concentration of the free sulphides increases in the ruminants digestive tract with increases in the level of dietary S, and this leads to the formation of insoluble CuS compound that can subsequently be excreted in the faeces (Suttle, 1974d). Grace *et al*. (1997) found contradictory result to those reported by Suttle (1974d) when S intake of grazing lambs were increased from 3.9 to 7.9 g/d (grazing pasture contained <0.5 mg Mo/kg DM). It was reported that the combined effect of Mo and S on Cu absorption is far greater than that of S alone (Suttle, 1974a; Gould and Kendall, 2011). Smart *et al*. (1986) reported that high S intakes might also reduce the Cu status of animals independently of Mo.

An equation was developed by Suttle and McLauchlin (1976) to predict the effects of dietary Mo and S on Cu availability of ruminants. This equation was accepted by ARC, (1980), and NRC, (2007) to predict Cu availability of sheep diets depending on Mo and S content. Data for the equation were derived from nine published experiments in which the true availability (TA) of Cu from semi-purified diets was predicted from monitoring plasma Cu responses, using the depletion-repletion technique described earlier. Dietary S and Mo levels in these experiments were within the normal ranges of pastures (1.0 - 4.0 g/kg DM and 0.5-4.5 mg/kg DM, respectively). The equation was also derived from unpublished experiment data involved lambs fed bruised-oats blood- meal diet, with a Mo concentration of 0.5 - 16.5 mg/kg and the liver Cu retention used as the measure of the TA.

$$\text{Log TA} = -1.153 - 0.0019\,(\text{Mo}) - 0.076\,(\text{S}) - 0.013\,(\text{Mo}\times\text{S}) \qquad \text{Equation 1.9.1}$$

However, Suttle (1978) presented another equation where the hepatic Cu retention data, used in equation 1.9.1, was omitted and data for 12 new diets were added:

$$\text{Log A}Cu = -1.113 - 0.0714\,\text{S} - 0.0187\,\text{S}\times\text{Mo} \qquad \text{Equation 1.9.2}$$

This equation was represented graphically to give a ready means of assessing the availability of Cu from diets of known S and Mo concentration (Figure 1.5). It was discovered later on that a difference as small as 3 mg Mo/kg DM and 0.5 g S/kg DM between two pastures is sufficient to reduce availability of Cu from 2.6 to 1.3% (Suttle, 1986).

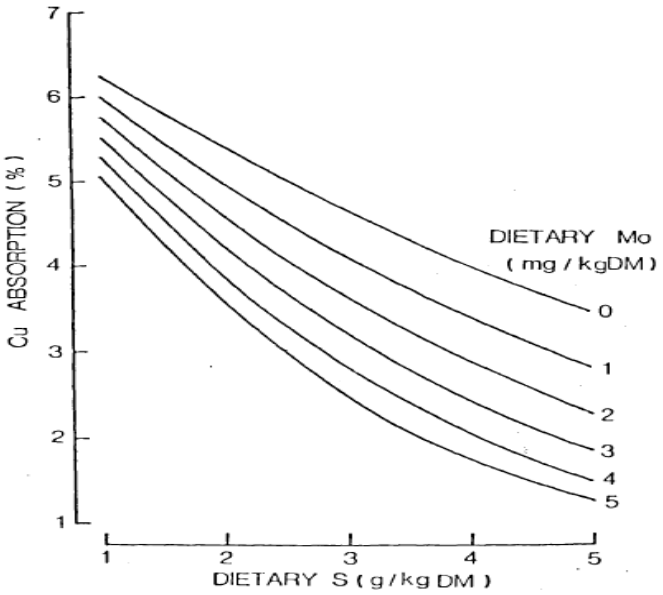

Figure 1.5. Effects of dietary molybdenum and sulphur concentrations on the efficiency of copper absorption in sheep given semi-purified diets (Suttle, 1978).

Animals obtain S and Mo from feedstuff and the normal concentration of these two minerals in normal animal diets ranges from 1 - 3 g/kg DM and from 1 - 5 mg/kg DM, respectively (NRC, 2005). At the upper end limits of these ranges a great reduction in Cu bioavailability and increased risk of Cu deficiency develop (NRC, 2005). Copper deficiency usually develop when forage Mo concentration exceeds 3 mg/kg DM and S concentration is about 5 g/kg DM, with Cu:Mo ratio in the feed of less than 2:1 (McDowell *et al*., 2001).

1.9.2. Effect of sulphur and iron interaction on copper

High dietary S has been found to reduce Cu availability significantly in ruminants through its effect on Cu absorption from the intestinal lumen or through involvement in the reaction with Mo to form different classes of TM in the rumen (Suttle, 1974; 1975; Bremner *et al*., 1987), see section 1.9.1. Bremner *et al*. (1987) observed a significant increase in the plasma and liver Cu concentration of calves when the dietary S reduced from 2.8 to 1.5 g/kg DM after 12 weeks when the dietary Fe was 100 mg/kg DM. Furthermore, removal of the supplemented S from the diet slowed down the rate of the daily liver Cu reduction of calves (0.025 vs. 0.0088 mg/kg DM). Bremner *et al*. (1987) suggested that Fe might exert an effect on Cu independently of S especially at high dietary Fe intakes. Similarly in sheep, Suttle *et al*. (1984) observed increases in the plasma Cu concentration of hypocupreamic Scottish Blackface ewes fed a diets supplemented with 10% soil on a dry matter basis, varying in Mo and Fe concentration but low in S (1 g/kg DM). Nevertheless,

when the same soils were added to diets high in S (4.1 g/kg DM), plasma Cu concentration decreased significantly after 21 days. Suttle *et al*. (1984) suggested that the soil constituent most likely to interact with Fe is S that reduces Cu absorption. Therefore, the authors proposed that Fe may inhibit Cu absorption through the temporary trapping of Fe as FeS by sulphide that can be absorbed rapidly through the rumen wall (Bray, 1969; NRC, 2001). Then, subsequently in the acidic abomasum environment, sulphide may be liberated to react with Cu forming acid un-absorbable CuS complex. In another experiment and when sheep fed a diet without any additional S supplement, Suttle *et al*. (1984) found that $FeSO_4$ was the only supplement that significantly reduced plasma Cu repletion of sheep irrespective of dietary S level.

1.9.3. Effect of high dietary iron on copper status of ruminants

Adequate Fe concentration is naturally supplied by forages which are the main source of feeding in grazing animals; Fe deficiency is unlikely to occur under the normal circumstances in grazing animals, but it may occur as a result of blood loss due to severe parasitic infestation or to other causes of haemorrhage (Givens *et al.*, 2000). High dietary Fe levels (>500 mg/kg DM; NRC, 2005) are frequently found in ruminant diets due to the naturally high Fe concentration in of the feedstuffs and to the contamination of feedstuffs with soil (Standish *et al.*, 1971). Feedstuff including alfalfa, soyhulls, and maize silage are often high in Fe (NRC, 1996; DePeters *et al.*, 2000). In the North-east of Scotland, Humphries *et al*. (1983) found that silages frequently contain 2000-4000 mg Fe/kg DM; that is considerably higher than the dietary Fe requirements of animals and the cattle fed these silages frequently become hypocupraemic. The Fe requirement of sheep is determined to be 30 - 50 mg/kg DM (NRC, 1980).

Most studies related with the Cu status of ruminants have focused on the antagonistic effects of Mo and S leading to the use of equations that predict Cu availability based solely on the presence of Mo and S (ARC, 1980; NRC, 2007). However, high dietary Fe has also been found to significantly reduce blood and tissue Cu levels of sheep (Prabowo *et al*., 1988; Williams, 2004) and cattle (Humphries *et al*., 1983; Bremner *et al*., 1987; Phillippo *et al*., 1987a). However, a limited number of studies have been conducted to determine the antagonistic effect of Fe on the Cu status especially in sheep.

There have been a limited number studies published to determine the effect of different levels of dietary Fe on blood, liver, and performance of cattle. For example Standish *et al*. (1969) conducted an experiment to study the effects of different levels of dietary Fe (0, 400, 1,600 and 3,200 mg/kg DM; as $FeSO_4$) on average daily live weight gain (ADWG), feed conversion efficiency (FCR) and mineral status of specific tissues of steer calves.

These researchers observed a substantial increase in the tissues Fe concentration of the calves given supplemental Fe diets (400 and 1600 mg/kg DM) after 84 days compared with those fed no Fe supplemental diet. On the other hand, increasing levels of dietary Fe were associated with reduced plasma and liver Cu concentration of animals. Liver Cu decreased with increasing rate of Fe inclusion of calves (260, 145, and 44 mg/kg DM) in groups supplemented with 0, 400, and 1600 mg Fe/kg DM, respectively, the basal diet contained 77 mg Fe and less than 0.5 mg Mo per kg DM. In another experiment to study the effect of Fe x Cu interaction, Bremner *et al*. (1987) designed a trial to determine whether the effects of Fe are dependent on a functioning rumen and via establishing dose-response relationships more precisely. These researchers also observed a significant reduction in the liver Cu stores of Friesian steer calves fed a diet supplemented with 0, 730, 1460 and 2190 mg Fe/kg DM (as $FeCl_3$) after 12 weeks. Nevertheless, dietary Fe levels had no effect on liver or plasma Cu concentration of the pre-ruminant calves. Campbell *et al*. (1974) also observed a rapid reduction in the hepatic Cu stores of grazing Friesian x Jersey weaners given Fe doses of 30 mg/kg live weight per day (as $Fe(OH)_3$), over a 205 days experiment period to investigate the effect of a moderately elevated intake of Fe on Cu status of calves.

Similar reductions in liver Cu stores were observed by Humphries *et al*. (1983) in Hereford-Friesian and Aberdeen Angus calves given diet supplemented with 800 mg Fe/kg DM, (as saccharated ferrous carbonate) compared with those given no supplementing Fe (19.2 vs. 72.2 mg/kg DM, respectively) after 8 weeks and to 3.6 vs. 72.0 mg/kg DM after week 32. Plasma Cu decreased by 50% of those given Fe supplements compared with no Fe supplemental calves after 12 weeks, and to 3.31 µmol/l in calves given supplemental Fe compared to 15 µmol/l in calves fed control diet at week 32. Phillippo *et al*. (1987a) found that hepatic Cu concentration was significantly higher at weeks 12 and 20 post-partum in cattle fed a control diet compared with those fed a diet supplemented with 500 mg Fe/kg DM (as saccharated ferrous carbonate). On the other hand, liver Fe concentration increased significantly in the group of heifers fed diets supplemented with Fe from week 8 to 32 compared with those fed control diet.

Johnson and Murphy (1988) observed a significant reduction in Cu absorption in rats fed a Cu deficient diet (0.42 mg/kg DM) supplemented with 191 mg Fe/kg DM after 20 days. Supplemental Fe increased faecal Cu concentration but reduced apparent Cu absorption from 1.5 to 0.9 mg/d, in the control and Fe groups, respectively. Yu *et al*. (1994) also observed a significant reduction in the liver Cu concentration of rats fed a purified diet containing 8 mg Cu/kg and 7, 40 or 389 mg Fe/kg in for 6 weeks period. In addition, apparent Cu absorption was reduced in Fe supplemented groups, 0.1448 mg/d vs 0.1042 mg/d in low and high Fe fed groups, respectively. Biliary excretion of Cu was also reduced

in the low and high Fe fed groups, 0.0273 mg/d, vs 0.0025 mg/d, respectively, and the biliary Cu excretion revealed a significant, positive correlation with liver Cu concentration of rats (Littledike and Young, 1993; Yu *et al*., 1994).

In sheep, Abdellatif (1968) reported that dietary Fe supplementation (2.6 - 5.2 g Fe/d) reduced Cu availability from 5.6 to 3.6%, and depressed hepatic Cu concentration significantly. However, no alteration in the blood Cu level was recorded. Prabowo *et al*. (1988) also observed a significant reduction in the liver Cu content but increases in the liver Fe concentration of growing lambs fed a forage-based diet with supplemental doses of Fe of 0, 300, 600, and 1200 mg/kg DM, (as ferrous carbonate) after 121 days. Significant reductions in plasma Cu level of lambs given 1200 mg Fe/kg were observed on day 56 of the experiment compared with lambs given diet supplemented with 600 mg Fe/kg (76 vs 69 µg/dl, respectively). Similarly, Williams (2004) observed a significant reduction in the liver Cu concentration of lambs given diets supplemented with 500 mg Fe/kg DM.

1.10. Effect of breed of sheep on copper metabolism

Breed has an important effect on Cu metabolism of sheep. Wiener and Field, (1966) were the first researchers who referred to the differences between sheep breeds in blood Cu concentration. Thereafter, their results were supported by other experimental results (Wiener *et al*., 1969; Wiener and Field, 1971a; 1971b; Wiener and Field, 1974; Wiener *et al*., 1987). This was particularly important to monitor the differences in the Cu requirements of different breeds and to investigate the Cu responsible disorders of sheep. Breed differences in Cu metabolism have also been reported in cattle; Angus cows and their calves had higher plasma Cu concentration than Simmental when given the same Cu containing diet (Ward *et al.*, 1995). Likewise, Mullis *et al*. (2003) found that Simmental steers had a lower Cu status compared with Angus steers. Mullis *et al*. (2003) have suggested a higher Cu requirement of Simmental compared with Angus. Higher biliary Cu excretion (about two fold) have been reported in Simmental heifers compared with Angus heifers fed the same diet (Gooneratne *et al*., 1994).

Wiener *et al.* (1978) observed a significant difference among different breeds of sheep (North Ronaldsay, Scottish Blackface and Welsh Mountain and crosses of these with the North Ronaldsay) in UK. Herbert *et al.* (1978) have observed that hepatic Cu retention of adult sheep, given a diet high in Cu, was 40% greater in Welsh Mountain than in Scottish Blackface sheep. This was associated with a greater plasma Cu concentration and a greater liver Cu retention of that breed (Woolliams *et al.*, 1983; Woolliams *et al.*, 1985). In another experiment, Hayter *et al*. (1973) investigated the effect of breed of sheep on plasma Cu concentration using Finnish Landrace, Merino and their reciprocal crosses;

Finnish Landrace were found to have significantly lower plasma Cu concentration followed by Merino, while the plasma Cu concentration of the crossbred was in midway of the parental breeds. Woolliams *et al*. (1983) observed a significant effect of sheep breed on liver Cu concentration when sheep were fed a diet supplemented with different levels of Cu (0, 9, 17 and 29 mg/kg DM as copper sulphate), liver Cu concentration was lowest in Scottish Blackface but Welsh Mountain had the highest, and the crossbred lambs had intermediate liver Cu concentration at 28 weeks. Similar observations were reported by Littledike and Young (1993), when they conducted a study to determine the effect of sheep breed on the Cu status of crossbred (F1); in this study the progeny of five ram breeds (Dorset, Finn sheep, Montadale, Romanov, and Texel) and two ewe breeds Rambouillet and a composite breed [l/2 Columbia, 1/4 Suffolk, 1/4 Hampshire] were used. Lambs were fed different Cu containing diets (5.2, 4.4, and 3.7 mg Cu/kg DM); Romanov and Finn sheep sired lambs had the lowest hepatic Cu concentration (307 and 327 mg/kg DM, respectively); Montadale- and Dorset sired lambs had intermediate liver Cu concentration (359 and 360 mg/kg DM, respectively), and Texel sired wethers had the highest liver Cu (458 mg/kg DM).

Miranda *et al*. (2006) reported that breed of cattle has a major influence on the liver Cu concentration. The average hepatic Cu concentration of Holstein Friesian (HF) was higher than that of Galician Blond (GB) calves (80.6 vs. 50.4 mg/kg wet weight, respectively), but GB x HF crosses had an intermediate liver Cu concentration (61.3 mg/kg). A similar trend was found in whole blood Cu concentrations where the mean blood Cu concentration of HF calves was greater than that of GB calves (14.02 vs. 11.77 µmol/l, respectively), with 13.05 µmol/l in GB x HF crosses. Jersey cows have been reported to be more vulnerable to Cu toxicity compared with Holstein Friesians cows (Du *et al.*, 1996). These genetic variations might be due to the efficiency of Cu absorption from the digestive tract (Du *et al*., 1996; Littledike *et al*., 1995), difference in the hepatic Cu storage and biliary Cu excretion, and endogenous Cu losses (Gooneratne *et al*., 1994), or maybe to the amount of ingested feed (Du *et al.*, 1996). In an experiment to determine the effects of two steer breeds (Simmental and Angus) and source of Cu and Zn on Cu and Zn status of steers fed a low Cu containing diet (3.5 mg/kg DM) supplemented with 1000 mg Fe/kg DM, as $FeSO_4$ (the basal Fe content of the diet was 334 mg/kg DM), Mullis *et al*. (2003) observed that liver Cu concentration decreased with time in both breeds. The authors also found that that Angus breed had higher liver Cu concentration than Simmental (14.9 vs 9.3 mg/kg DM, respectively) and had a higher Cp activity than Simmental (0.3 vs 0.15 mg/l, respectively).

Lüke and Wiemann, (1970) reported that Texel sheep had a higher liver Cu concentration (1206 mg/kg DM) than East Friesian (633 mg/kg) or German Blackhearted Mutton (557

mg/kg) when fed the same diet. Lüke and Wiemann, (1970) have reported that Texel sheep was the only breed that died from chronic Cu poisoning. Woolliams *et al*. (1982) found that Texel x Blackface crossbred lambs had more than twice the liver Cu concentration of pure Blackface lambs when fed a diet containing 12 mg Cu/kg DM (695.6 vs 291.6 mg/kg DM, respectively) or 20 mg Cu/kg DM (1491.5 vs. 567.1 mg/kg DM, respectively) after 13 weeks. Furthermore, the researchers found that the Cu absorption in Texel-cross was twice of that of pure Scottish Blackfaces (0.137 vs. 0.056 mg/kg DM, respectively) and the liver Cu concentration increased in Texel-crossbred lambs (receiving a diet containing 20 mg Cu/kg DM) from 28.9 to 1491.5 mg/kg DM after 13 weeks. Woolliams *et al*. (1982) also found a similar increases in the kidneys Cu concentration of lamb given a high Cu diet (20 mg/kg) compared to group fed diet containing 12 mg Cu/kg DM.

In housed lambs, fed a diet containing 6.1 mg Cu/kg DM, Suttle *et al*. (2002) observed that Texel's had a higher liver Cu concentration compared with Suffolk lambs. Suttle *et al*. (2002) have found that the increases in liver Cu concentration in the last 4 weeks was less than 2% in Suffolk compared with nearly 30% in Texel lambs. After 26 weeks, Texel's had 1.4-times higher liver Cu concentration (667.12 mg/kg DM) than those of Charollais (462.93 mg/kg DM) and Suffolk, (436.56 mg/kg DM).

1.11. Copper requirements of animals

The published dietary Cu requirements of sheep have changed over time. Copper requirements of sheep had been set at the level of 5 mg/kg DM (NRC, 1975) then increased to 7 – 11 mg/kg DM (NRC, 1985). However, ARC (1980), depending on the physiological status of sheep, has estimated the dietary Cu requirements of sheep to range from 1 – 8.6 mg/kg DM. Based on the absorption coefficients of 0.06, 0.03, and 0.015 for roughage, green pastures, and Mo rich pastures, Underwood and Suttle (1999) estimated Cu requirements of 4.3 – 28.4 mg/kg DM. In NRC (2007) the estimates of sheep Cu requirements were determined in a factorial approach, and three equations were developed, for growing lambs, gestating and lactating ewes, utilising the information presented in ARC (1980) and Grace and Clark (1991). The minimum recommended Cu requirements have increased over the years as a result of the better understanding of the interactions of Cu and its antagonists. For example, the Cu requirement of beef cattle in NRC (1976) was 4 mg compared with 10 mg/kg DM in NRC (1996). The minimum dietary requirement of Cu for neonatal pigs is 6.0 mg/kg DM, but dietary Cu levels of up to 250 mg enhance growth rate at least at the growing phase (Puls, 1988). The recommended Cu level of diets containing about 0.25% S and 2 mg Mo/kg DM is 10 mg Cu/kg DM (NRC, 2000), and this amount should be enough to prevent any antagonistic effects between

these minerals (NRC, 1996). Smart *et al*. (1986) found that 10 mg of dietary Cu was not sufficient to maintain liver Cu stores of pregnant Herford cows and heifers receiving sulphated water (500 mg S/l).

Under current EU regulation, the maximum allowed Cu level in sheep diets is 17 mg/kg on a DM basis (EFSA, 2015). The accurate determination of individual animal Cu requirements is calculated as below:

$$\text{Dietary Cu requirement} = \frac{\text{Net requirement}}{\text{efficiency of absorption}}$$

Equation 1.11.3

Predicted absorption coefficients present in Table 1.7, are used by NRC (2007) who formulated a series of equations to estimate the Cu requirements of sheep depending on their physiological status. Estimated Cu absorption coefficient (A_C) of lambs, pregnant and lactating ewes according to ARC (1980), depends on Mo + S but does not take into account Fe effects, are present in (Table 1.7). As sheep age increase, the A_C of Cu declines; consequently, A_C become lower in older sheep compared with the young and pre-weaned lambs.

Table 1.7. Absorption coefficient (A_C) of Cu of sheep at different ages and physiological statuses (Adapted from ARC, 1980; NRC, 2007)

	Absorption Coefficient (A_C)
Pre-weaned lamb	
5 kg	0.90
10 kg	0.53
20 kg	0.20
Weaned lamb	
Pasture	0.045
Feedlot	0.06
Ewe	
Gestating	0.06
Lactating	0.045

Recently Suttle (2010) published updated estimates of Cu requirements of sheep as listed in Table 1.8. However, breed of sheep can affect Cu absorption and this should be taken into consideration when estimating Cu requirements. Woolliams *et al*. (1983) reported that variation in the efficiency of Cu absorption indicates that Scottish Blackface ewes have a greater Cu requirement than Welsh Mountain ewes. This is the likely explanation of the

greater occurrence of swayback and the lower whole blood concentrations of Cu detected in the Blackface sheep, compared to Welsh, in a single grazing flock under same conditions (Wiener and Field, 1971a). Breed of sheep affects Cu absorption and metabolism; however, the estimated data in Table 1.8 did not consider breed effects.

Copper requirements of ruminants are governed by species, mineral composition of the diet, infection, and physiological status of the animal (Suttle, 2004, 2005; Soetan *et al*., 2010). Most forage contains Cu at levels equal to or above the NRC requirements for ruminants. However, as plants mature and the phytate and lignin content increases, Cu bioavailability decrease; and it has also been reported that Cu in cereals and cereal by-products is absorbed more readily than from grazed herbage (Minson, 1990). The ability of any feedstuff to meet Cu requirements of animal or to cause poisoning is mostly depend on Cu absorption rather than Cu concentration in the diet (Suttle, 2010).

Table 1.8. Estimates of the dietary Cu requirements of sheep fed three diets with contrasting Cu absorbabilities

	Live weight (kg)	Growth rate/milk yield (g/day)	Net requirement (mg/day)	DM intake (kg/d)	Gross requirement (A_{Cu}) (mg/kg DM)		
					0.06	0.03	0.15
Lamb	20	100	0.13	0.50	4.3	8.6	17.2
		200	0.18	0.70	4.3	8.6	17.2
Ewe 16-week twin foetuses	75	0	0.63	1.5	7.0	14.0	21.0
Lactating ewe	75	1000	0.52	1.5	5.8	11.6	23.2
		3000	1.24	2.9	7.1	14.2	28.4

Adapted from: Suttle (2010)

A great deal of evidence has shown that the presence of high dietary concentrations of both Mo and S form TM that react with Cu in the rumen, forming highly stable insoluble compounds that cannot be digested and absorbed (Suttle, 1991; Ward *et al*., 1993; Gould and Kendall, 2011). When the concentration of both Mo and S is high, Cu availability reduces to less than 0.01 compared with more than 0.04 in diets low in the both antagonists (Minson, 1990). Absorption of Cu has also been found to decrease in the presence high dietary iron (Campbell *et al*., 1974; Humphries *et al*., 1983).

Generally Cu requirements increase as the dietary levels of Mo, S and Fe increase (Suttle, 2010). Therefore, it is important to keep Cu and Mo at a certain ratio. The

minimum Cu:Mo ratio in beef cattle diets is 3.0, and an adequate ratio is 4.3, and the ideal ratio from 6.0 -10.0 (Puls, 1988). The Cu:Mo ratio in sheep is not fixed, but declines from 5:1 to 2:1 as pasture Mo concentrations increase from 2 to 10 mg/kg DM (Suttle, 1991). Miltimore and Mason, (1971), reported that the critical Cu:Mo ratio in feedstuff appears to be 2:1, and pastures or feeds with a lower ratio produce secondary Cu deficiency. Alloway (1973) suggested a Cu:Mo ratio of no less than 4:1 to ensure that the Cu requirement will be met.

1.12. Conclusion

The importance of copper deficiency on sheep health and performance has been investigated in numerous studies, but most of the focus has been on the effect of the antagonists sulphur and molybdenum. Nevertheless, the effects of high dietary iron as an important copper antagonist in sheep is not well investigated and the mode of action still requires further investigation. Different authors have reported conflicting results, and have suggested different hypothesis to explain the impact of elevated dietary iron intakes on copper status of sheep. The objectives of this study were to investigate the effect of level of dietary iron with and without supplemented sulphur on copper status of sheep, as well as to study the effect of iron supplementation of different breeds on copper status and performance of growing lambs. The overall null hypothesis for this work is that iron and breed do not affect Cu metabolism in sheep.

CHAPTER 2

General Materials and methods

2.1. Proximate analysis of samples

All samples were analysed in duplicate at Harper Adams University labs using methods in accordance with AOAC (2016).

2.1.1. Dry matter determination (DM)

Dry matter content of the diets and faecal samples were determined according to Association of Official Analytical Chemists (DM, AOAC; 930.15). Sub samples were accurately weighed and oven dried (Binder, Cole-Palmers, UK) at 105°C overnight or freeze dried (Edwards Modulyo freeze dryer, Sussex, UK) until stable weight. Samples were cooled in a desiccator after being taken from the oven and reweighed. The DM was then calculated:

$$\text{DM (g/kg)} = \left[\frac{\text{Weight of dried sample (g)}}{\text{Weight of fresh sample (g)}}\right] \times 1000$$

Equation 2.2.4

2.1.2. Ash and Organic matter determination

Dried and milled feed and faecal samples were analysed for ash and organic matter (OM) according to AOAC (ash, AOAC; 942.05). Approximately 2 g of dried samples were accurately weighed into pre-weighed labelled porcelain crucibles. Samples were ashed in a muffle furnace (Gallenkamp Muffle Furnace, Size 3, GAFSE 620, Gallenkamp, Loughborough, UK) for 4 h at 550°C, cooled in a desiccator and reweighed. Ash content was calculated:

$$\text{Ash (g/kg DM)} = \left[\frac{\text{Weight of ash (g)}}{\text{Weight of dry sample before ashing (g)}}\right] \times 1000$$

Equation 2.2.5

Organic matter (OM) of the samples were calculated as 1000 minus ash content (g/kg DM).

2.1.3. Crude protein determination (CP)

Dried feed and faecal samples were analysed according to Association of Analytical Chemists (CP, AOAC, 2016, 968.06) by the use of an element auto analyser LECO FP528 (LECO Corp, Stockport, UK) operating the Dumas method. Approximately 150 mg of dried ground sample was accurately weighed into aluminium foil squares. This was then placed into the auto analyser, CP content of samples were calculated:

CP (g/kg DM) = Nitrogen content (g/kg DM) X 6.25 Equation 2.2.6

2.1.4. Ether extract (EE)

The ether extract content of feed samples was determined according to a solvent method of FOSS (1987) using Soxtec apparatus (HT 1043 extraction apparatus, FOSS, Warrington, UK). Approximately 1 g of dried ground sample was weighed accurately into a cellulose extraction thimble (Whatman Plc, Maidstone, UK). The thimbles were then plugged with de-fatted cotton wool and fitted in the extraction unit. Total fat was extracted in a pre-weighed extraction cup after boiling of the feed samples in 25 ml of cold petroleum ether (Fisher Scientific, UK) for 1 h. Samples were then removed from the solvent and rinsed for an additional 15 min. The final traces of the solvent were evaporated off, and the extraction cups removed from the apparatus and moved into a fume cupboard. After the extraction cups were cooled, reweighed and the EE determined:

$$\text{EE (g/kg DM)} = \left[\frac{\text{Weight of fat (g)}}{\text{Weight of dried sample (g)}}\right] \times 1000$$

Equation 2.2.7

2.1.5. Neutral detergent fibre (NDF)

The neutral detergent fibre (NDF) of dried feed and faeces samples were determined according to the method of Van Soest *et al*. (1991) using Fibertec apparatus (1020, FOSS, Warrington, UK). The NDF reagent, was previously prepared from mixing 93 g of di-sodium ethylene diamine tetra acetic acid dehydrate (EDTA), 34 g sodium tetra borate ($Na_2B_4O_7.10H_2O$), 150 g sodium dodecyl sulphate (SDS), 50 ml tri-ethylene glycol, and 22.8 g anhydrous disodium hydrogen phosphate (Na_2HPO_4) to make 5L solution with distilled water and the pH adjusted to approximately 6.9 to 7.1. Alpha amylase solution

was prepared by dissolving 2.8 g of α-amylase (α-1, 4-glucan 4-gluconohydralase, Enzyme # 3.2.1.1 ~80EU/mg) from *Bacillus subtilis spp* (Sigma, Gillingham, UK) in 90 ml of distilled water followed by the addition of 10 ml of tri-ethylene glycol.

Approximately 0.4 to 0.6 g of dried ground samples was accurately weighed into glass crucibles (porosity 1, Soham Scientific, Ely, UK). Crucibles were tightly fitted onto the Fibretech® 1020 hot and 1021 cold extractor (Foss UK Ltd, Cheshire,UK) making sure the valves were in the closed position. Cold neutral detergent reagent (25 ml) followed by a few drops of octanol, reagent grade (Sigma, Aldrich, Dorset, UK) were added to each of the samples. The heat control knobs were turned to full and as samples started boiling, the heat was reduced. Samples were digested for 30 min then the heat was switched off. Another 25 ml of cold neutral detergent reagent and 2 ml of α-amylase solution were added and the samples brought to the boil and digested for an extra 30 min. Samples were then filtered and washed with 20-30 ml of hot distilled water (80°C). A further 2 ml of α-amylase solution and 25 ml of hot distilled water were added to the samples and allowed to stand for 15 min. Samples were then filtered and washed 3 times with hot distilled water, the crucibles removed from the Fibertech® hot and cold extractor and dried overnight at 105°C. After cooling in a desiccator, crucibles were weighed and then placed in a muffle furnace at 550°C for 4 h. Crucibles were then allowed to cool in a desiccator to room temperature and reweighed:

NDF (g) = (crucible + dry fibre weight) – (crucible + ash weight)

$$\text{NDF (g/kg DM)} = \left[\frac{\text{NDF weight (g)}}{\text{Weight of dried sample (g)}}\right] \times 1000$$

Equation 2.2.8

2.1.6. Gross energy determination (GE)

To determine the GE of the feed and faecal samples, an adiabatic bomb calorimeter (Parr 6200 Instrument Company, Moline, IL, 61265, USA) was used. Dried ground samples were pelleted using a 2811 Parr pellet press (Parr instrument Co., Moline. USA) and accurately weighed. Pelleted samples were then placed into a crucible. About 10 cm of fuse wire was inserted through the holes of the bomb; care was taken to ensure the wire did not contact with the sample. The apparatus was assembled, filled with O_2 gas, and placed in a bucket containing 2 L of water and the wires connected. The bomb calorimeter measured the samples energy content by burning it with O_2 under enclosed conditions at a constant volume. The energy produced was measured as MJ/kg DM.

2.2. Blood sampling

Blood samples were collected via jugular venepuncture using a 21G x 1″ (0.8 x 25 mm) needles in to Vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK). Vacutainers (4.0 ml) containing K_2EDTA (for samples used to determine whole blood haematology and SOD activity), (6.0 ml) silica (for samples used to determine ceruloplasmin), or lithium heparin (for samples used to determine mineral concentration in chapter 3) and sodium heparin (for samples used to determine mineral concentrations).

Blood collected in lithium heparin or sodium heparin vacutainers were centrifuged at 1000 xg for 10 minutes at 4°C in Rotina 46R, Hettich Zentrifugen centrifuge (Anderson Hettich GmbH & Co, Tuttlingen, Germany). Plasma was removed using disposable pipettes and stored in labelled micro-tubs at -20°C. Silica vacuum tubes were stored at 4°C for 24 h before centrifuging (1000 xg for 10 minutes at 4°C) and subsequent removal of serum, which was stored at -20°C for subsequent analysis. The K_2EDTA containing vacuum tubes were directly analysed for whole blood haematology (Woodley Equipment Company Ltd., Bolton, UK) and a subsample of whole blood was collected into micro-tubes using disposable plastic pipettes and stored at −20°C for the determination of SOD activity.

2.3. Haematology profile

Following blood sampling, the EDTA Vacutainers containing whole blood samples were thoroughly mixed using a Spiramix 5, (Denley Instruments Ltd., West Sussex, UK) for 15 minutes. For the Vet Animal Blood Counter (Woodley Equipment Company Ltd., Bolton, UK) calibration, a haematology control sample (ABX Minotrol 16; Horiba ABX Diagnostics, Bedfordshire, UK) was used prior to the start of analysis each time to ensure the machine results were accurate. The whole blood samples were analysed for white blood cell count (WBC), red blood cell count (RBC), haematocrit (HCT) and haemoglobin concentration (Hb).

2.4. Superoxide dismutase activity (SOD)

The activity of SOD was determined using an adapted method of Misra and Fridovich (1977) for use on a Cobas Mira Plus blood analyser (Ransod SD125, Randox Laboratories, County Antrim, UK) using a Ransod kit. The method employed xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The activity of SOD was then measured by the degree of inhibition of this reaction (Randox Laboratories, Ltd). Frozen EDTA blood samples were defrosted (lysed cells) and vortexed using a MT-20 vertex-mixer (Philips Harris Ltd., Shenston, UK). A volume of 250 µl of

whole blood was accurately pipetted into a 1 ml micro-centrifuge tube (Sarstedt Ltd., Leicester, UK), and an additional 0.750 ml of purite water was added. The sample was vortexed and 10 µl of sample was then added to 490 µl of 0.01 mol/l phosphate buffer, pH 7.0 (Ransod, Randox laboratories, County Antrim, UK) into a micro-centrifuge tub and vortexed thoroughly. Samples were then carefully transferred into Mira cups (ABX Diagnostic, Bedfordshire, UK), and placed into the reagent racks and analysed by an automated method on Coba Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). SOD activity (U/g Hb) calculated:

$$\text{SOD (units/g Hb)} = \frac{\text{SOD units/ml of whole blood}}{\text{g Hb/ml}}$$

Equation 2.2.9

A control sample (Ransod, Randox laboratories, County Antrim, UK) was analysed to confirm that it was within the expected range each time before running the samples.

2.5. Ceruloplasmin activity

The activity of Cp was determined according to the adapted method of Henry *et al*. (1974) for the use on Cobas Mira Plus (ABX Diagnostics, Shefford, Bedfordshire, UK). Capability of Cp to act as a general oxidase was employed, where Cp oxidases 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 serum, a blank (CPB) was run in which sodium azide inhibits the Cp activity, and the results subtracted from the test (CPT). Individual serum samples were pipetted into Mira cubs (ABX Diagnostics, Shefford, Bedfordshire, UK) and placed in the required reagent rack on the Cobas Mira Plus. A 0.1M solution of PPD (BDH Laboratory Supplies, Poole, Dorset, UK) was prepared in 100 ml of 0.1M acetate buffer and adjusted to pH 6.0. Sodium azide (BDH Laboratory Supplies, Poole, 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 PPD solution pH 6.0. a blank (CPB) reagent was prepared by adding 10 ml acetate buffer pH 6.0, 10 ml PPD solution pH 6.0 and 10 ml sodium azide solution. The activity of the Cp (mg/dl) was calculated as:

CPT - CPB = Ceruloplasmin activity (mg/dl)

2.6. Concentrate and faecal minerals

Feed and faecal samples were dried to a constant weight at 105°C (section 2.1.1) and ground with a Delongh KG 79 (Freemans PLC, Sheffield, UK) to pass through a 1 mm mesh. A total weight of 0.5 g dried, milled sample was accurately weighed into a 50 ml DigiTUBE and 1 ml of concentrated analytic reagent grade HCl (37%, Fisher Scientific., UK) and 6 ml of concentrated analytic reagent grade HNO_3 (70%; Fisher Scientific., UK) added. The tube containing sample and reagents were then digested using the DigiPREP (Qmx Laboratories., Thaxted, Essex, UK) as described by Cope *et al*. (2009). The tubes were heated in the DigiPREP 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. The samples were cooled down to room temperature, and then diluted with purified water (Purite Ltd, Thame, UK) before being analysed by inductively coupled plasma-mass spectrometry model ICP-MS (Thermo Fisher Scientific Inc., Hemel Hempstead, UK). Digested feed and faecal samples were analysed for S, Fe, Cu, Zn, Mn and Mo following dilution in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK). To monitor consistency and reliability of readings, a certified EU reference samples of hay (BCR-129) and liver (BCR®-185R) were routinely extracted and analysed as described by Cope *et al*. (2009).

2.7. Plasma minerals

Before analysis, plasma samples were defrosted at room temperature or defrosted overnight at 4°C. Then the samples were thoroughly mixed using a MT-20 vortex-mixer (Phillip Harris Ltd., Shenton, UK) and centrifuged at 13000 xg for 30-second using an eppendorf mini centrifuge (Eppendorf centrifuge 5415 D., Hamburg, Germany). Prior to analysis, plasma samples were diluted 1:20 in 0.5% HNO_3 (70%; Fisher Scientific., UK) 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). Samples then were thoroughly vortex mixed and the analysis was carried out using a calibration graph at concentration levels of 0, 50 µg/kg, 250 µg/kg, 500 µg/kg, 2500 µg/kg and 5000 µg/kg in blank, standard 1, standard 2, standard 3, standard 4, and standard 5, respectively, for Fe, Cu, Zn. While for Mn and Mo standards concentrations of 0, 5, 25, 50, 250, 500 µg/kg were used in blank, standard 1, standard 2, standard 3, standard 4, and standard 5, respectively. All samples were then analysed by ICP-MS (Thermo Fisher Scientific Inc., Hemel Hempstead, UK) to determine the level of minerals in plasma.

2.8. Tissue mineral determination

After lambs had been slaughtered, fresh liver samples (and kidney samples in experiment 1) were collected, into clean plastic zipped bags and stored at -20°C for subsequent analysis. Approximately 250 mg of fresh liver or kidney sample were weighed accurately into a 50 ml tube. Samples were oven dried overnight at 60°C and then were cooled in a dessicator and reweighed to determine the DM content of the samples. Dried samples were digested by adding 6 ml of HNO_3 (70%; Fisher Scientific., UK) to each tube using an automated dispenser and the caps replaced and placed in an oven overnight at 60°C. Samples were then removed from the oven and cooled in the fume cupboard and diluted to 50 ml with purite water. Digested samples were then thoroughly mixed by shaking and stored in a fridge until ready for analysis by ICP-MS. Tissue samples were analysed by ICP-MS for Cu, Fe, Mn, Zn and Mo after diluted (1:20) in 2% HNO_3, 1% methanol, and 0.1 Triton X-100 as described by Cope *et al*. (2009).

2.9. Plasma trichloroacetic acid (TCA)-soluble copper

Plasma samples were defrosted at room temperature or left in a fridge overnight to defrost. Then the samples were thoroughly mixed using a MT-20 vortex-mixer (Phillip Harris Ltd., Shenton, UK) and centrifuged at 13000 rpm for 30 second using an eppendorf mini centrifuge (Eppendorf centrifuge 5415 D., Hamburg, Germany). Plasma samples of 0.5 ml were placed into an eppendorf tube and 0.5 ml of 10% trichloroacetic acid (TCA) solution (Sigma-Aldrich Co., Germany) added and vortex mixed. Samples were left at room temperature for 5 minutes then centrifuged at 1500 g for 10 minutes. A volume of 0.5 ml of supernatant was pipetted into an empty auto sampler tube. Using an automated dispenser (Hamilton Microlab model 510B Hamilton Microlab Co., UK) 4.50 ml of the plasma diluting acid (2% NHO_3, 1% methanol, and 0.1% Triton X-100) containing the internal standard Gallium (Romil Ltd., Cambridge, UK) was added (Cope *et al*., 2009). Samples were vortex mixed before being analysed by ICP-MS.

2.10. Live weight determination

Lamb live weight was recorded once a week using a weigh crate (Shearwell Data Ltd., Somerset, UK), and for precision and accuracy was calibrated using standard weights prior to weighing (F.J. Thornton and Co. Ltd., Wolverhampton, UK).

CHAPTER 3

Effect of different levels of dietary iron on copper metabolism of growing sheep

3.1. Introduction

Iron is an important microelement required for normal body functions (Backe *et al.*, 2016). However, its requirements vary depending on LW, age, diet composition and the condition for which it is used (NRC, 2005; Humann-Ziehank, 2016). Needs in terms of dietary Fe concentration will decline as individuals of all species grow to mature body weight (Suttle, 2010). Iron deficiency is of limited practical significance in most livestock, but examples of situations in which animals are vulnerable to Fe deficiency are calves raised for veal, Cu-supplemented pigs, and animals with parasitic infection (Underwood and Suttle, 1999). The Fe requirement of animals ranges from 50 - 100 mg/kg DM depending on age and species (NRC, 2005). However, many feedstuffs such as alfalfa, soyhulls, and maize silage, can provide significantly higher levels of dietary Fe for example forages contain over 700 mg Fe/kg DM (Standish *et al*., 1971; NRC, 1996; DePeters *et al*., 2000).

It is well documented that dietary Fe can greatly reduce Cu availability in ruminants when present in excess amounts (Gengelbach *et al.*, 1994; Humphries *et al.*, 1983; Phillippo *et al.*, 1987b; Prabowo *et al.*, 1988; Standish *et al.*, 1969; Williams, 2004). Research has indicated that high Fe levels reduced Cu status of sheep (Prabowo *et al*., 1988; Williams, 2004), cattle (Koong *et al.*, 1970; Standish *et al*., 1971; Campbell *et al*., 1974; Humphries *et al*., 1983; Bremner *et al*., 1987; Phillippo *et al*., 1987a; Gengelbach *et al*., 1994; Chase *et al.*, 2000) and goats (Schonewille *et al*, 1995).

Excess dietary Fe can adversely affect performance of ruminants and utilization of Cu and also affect Zn and Mn (Coup and Campbell, 1964; Standish *et al*., 1969; 1971; Standish and Ammerman, 1971; Campbell *et al*., 1974; Humphries *et al.*, 1983). To study the effect of Fe level on blood and tissue Cu of cattle, several studies have been conducted and in most of these studies the focus was on using one or two levels of dietary Fe (Standish *et al*., 1971; Humphries *et al.*, 1983; Phillippo *et al*., 1987b; 1978a; Gengelbach *et al*., 1994; Chase *et al*., 2000). Few researcher have studied the effects of 4 levels of dietary Fe on Cu status and performance of cattle (Bremner *et al*., 1987; Koong *et al*., 1970; Standish *et al*., 1969). In sheep, few studies have been conducted to investigate the effect of dietary

Fe on Cu status and the main focus was on using two levels of Fe (Abdellatif, 1968; Rosa *et al*, 1986; Williams, 2004). Most of the studies in sheep regarding Cu antagonists were conducted to investigate the effects of Mo and S antagonists and less attention have been paid to the effect of Fe.

Suttle and McLauchlin (1976) proposed an equation to predict the dietary Cu availability in ruminant diets based on the relationship between S and Mo and their concentration in the diet. The equation was widely accepted by both ARC (1980) and NRC (2007). However, Van Ryssen *et al.* (1986) indicated that the equation might have some limitations to predict Cu availability when dietary Cu is high; in addition the equation did not have any consideration to the antagonistic effect of Fe on Cu availability (NRC, 2005). Little is known about the mechanisms whereby Fe affect Cu metabolism of sheep and it is unclear whether the effect of Fe is on the absorption or the hepatic retention of Cu. Therefore, two studies were conducted at Harper Adams University (HAU) to determine the influence of different levels of dietary iron on performance characteristics, mineral composition of liver, kidney, blood, and apparent mineral absorption in growing lambs.

3.2. Materials and methods

All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by the Harper Adams University Research Ethics Committee before initiation of the experiment.

3.2.1. Animals and experimental design

Experiment 1:

Forty weaned Texel-cross lambs (Suffolk x North of England Mules ewes mated to Texel rams), 20 wethers and 20 female, with an initial live weight (LW) of 33.2 ± 0.4 kg were used in a 6 weeks study. Prior to the commencement of the experiment the lambs were blocked by LW and sex then 4 lambs were chosen and slaughtered by stunning and exsanguination in a commercial abattoir to determine the initial liver minerals concentration of lambs (first slaughter group). Whole livers and one kidney per lamb were collected and stored at -20°C until analysed (Table 3.1).

Table 3.1. Mean liver minerals concentration of lamb slaughtered on day 0 (mg/kg DM) (n =4, ±SD)

Cu	Fe	Mn	Mo	Zn
313.6 ±136.99	339.8 ±28.95	15.1 ±1.18	1.4 ±0.33	107.1 ±7.59
(441.5, 121.5)[a]	(372.8, 311.2)	(16.7, 14.0)	(1.7, 1.0)	(118.1, 101.2)

[a](Maximum value, Minimum value)

The remaining 36 lambs were then randomly allocated to 1 of 4 dietary treatments (9 lamb per treatment) also by LW and sex. Lambs were housed individually in metal pens and bedded on wood shavings throughout the experiment.

3.2.2. Diet formulation and treatments

The raw feed ingredients were chosen based on their published low Cu content (MAFF, 1992). The diets were mixed on farm using a Keenan mixer wagon (Richard Keenan Ltd., Warwickshire, UK) after calibration to ±1 kg (Table 3.2). The basal diet was formulated to supply 11.14 MJ/kg DM metabolisable energy (ME) and 93.82 g/kg DM metabolisable protein (MP). The formulated basal diet was predicted to supply 8.92 MJ/kg DM of FME (Table 3.3), required for lambs weighing 30 kg to grow at 200 g/day (AFRC, 1993).

Table 3.2. Feed ingredients composition of the basal diet (g/kg)

Ingredient	Quantity
Dried grass nuts	500
Barley rolled	200
Sugar beet pulp	85
Soybean meal	110
Molasses	50
Megalac	30
Mins/vits premix [1]	25

[1] Mineral premix (25 kg/ ton) (RUMENCO LTD., Burton upon Trent, UK) containing 320,000 IU/kg Vit A, 100,000 IU/kg Vit D3, 2,000 IU/kg Vit E, 18.5% calcium, 2.0% phosphorous, 1.0% magnesium, 12.0% sodium, 25% chloride, 20 mg/kg selenium, 90 mg/kg cobalt, 150 mg/kg iodine, 3000 mg/kg manganese, and 3000 mg/kg zinc.

The mineral composition of the experimental diets was determined by (ICP-MS) using the method described by Cope *et al*. (2009) (see section 2.5).

Table 3.3. Chemical composition of the basal diet of experiment 1

Item	Treatments			
	C[1]	L[2]	M[3]	H[4]
DM (g/kg)	885.3	885.8	877.0	875.4
CP	197.9	192.6	180.8	196
EE	29.2	22.4	27.0	29.0
NDF	377.4	320.6	367.2	330.1
Ash	100.8	96.3	96.3	103.4
OM	899.2	903.7	903.7	896.6
NCDG[1]	763.3	794.0	769.8	783.7
ME (MJ/kg DM)[2]	11.42	11.68	11.45	11.70
Mineral concentration (mg/kg DM)				
Cu	9.9	8.0	7.2	8.8
Fe	487.6	693.7	953.1	1399.8
Mo	2.5	2.1	2.1	2.3
Mn	137.5	130.6	144.6	147.5
Zn	97.8	111.0	102.1	127.1
S (g/kg of DM)	4.2	4.2	3.8	4.3

[1] Neutral cellulose gamanase digestibility.
[2] Metabolisable energy calculated using equation provided by AFRC (1993).

Dietary S was supplemented to C, L, M, and H diets (1.77, 1.66, 1.55 and 1.43 g S/kg DM, respectively) as $(NH_4)_2SO_4$ (West Bromwich Essex, UK) to achieve a total concentration of 4.0 g S/kg DM across all dietary treatments. The Fe was supplemented as $FeSO_4.7H_2O$ (Fisher Scientific., Leicester, UK) (Table 3.4). The S coming from $FeSO_4$ supplements was accounted for before mixing the diets. Supplemental feed grade urea (Trouw Nutrition,

Northwich, Cheshire, UK) was used to balance the N levels of the diets based on the N content of $(NH_4)_2SO_4$ inclusion.

Table 3.4. Dietary treatments used in the study

	Treatments ID			
	C[1]	L[2]	M[3]	H[4]
Supplemental Fe (mg/kg DM)	0	250	500	750

[1]Control diet (no Fe supplements), [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

3.2.3. Experimental routine

Lambs were fed at a restricted level to support a predicted live weight gain (LWG) of 200 g/d (AFRC, 1993). Feed was weighed daily using metric scales and offered twice a day at 08:30 and 16:30h into individual clean plastic feed buckets, to avoid mineral contamination with *ad-libitum* access to clean water. Feed samples (~1 kg) were collected once a week and feed refusals collected twice a week to determine the total DM intake.

3.2.3.1. Live weight determination

Live weight of lambs was recorded weekly on a Monday at 11:00 h using a weight crate (Shearwell Data Ltd., Somerset, UK). Metric standard weights were used to calibrate the scale at each weighing (F.L. Thornton and Co. Ltd., Wolverhampton, UK). Weekly lamb weights were used to calculate the daily feed allowance the following week to achieve a daily live weight gain (DLWG) of 200 g/d (AFRC, 1993).

4.2.3.2. Blood sampling and analysis

Jugular blood was collected at 11:00 h in weeks 0, 1, 2, 3, 4, and 6 for plasma, serum, and whole blood. Blood was collected in heparinised vacuum tubes (coated with lithium heparin) for trace mineral analysis (Becton Dickinson Vacutainer systems, Plymouth, UK). To collect plasma, blood tubes were centrifuged at 1000 ×g for 10 min at 4°C (Sigma Laboratory Centrifuges., Germany). Plasma was then removed and stored at -20°C until subsequent analyse. Whole blood samples collected in vacutainer, spray coated with K_2EDTA (Becton Dickinson Vacutainer systems, Plymouth, UK) were analysed for WBC, RBC, Hct and Hb using a Vet Haematology Analyser device (Woodley Equipment Co Ltd., Bolton, UK) as described in section 2.3. Subsamples of the whole blood were taken and stored at -20°C for SOD activity determination using a Cobas Mira Plus auto-analyser

(ABX Diagnostics, Bedfordshire, UK) as described in section 2.4. Vacuum tubes for serum collection (silica coated) were stored overnight at 4°C before being centrifuged at 1000 ×g for 10 min at 4°C, then serum was removed and stored at -20°C for Cp activity determination based on the method of Henry *et al*. (1974), using Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK), see section 2.5.

3.2.4. Tissue sample collection and analytical procedures

At the end of week 6, all lambs were sent to a commercial abattoir and slaughtered using the same procedure of killing used for the first lamb group. Whole livers and one kidney were collected immediately after the lambs were slaughtered. Liver weight was recorded and a sample of approximately 35 g was taken and stored at -20°C until subsequent analysis. Weekly concentrate samples were bulked and oven dried (Binder, Cole-Palmers, UK) at 105°C overnight before being milled (see section 2.1.1). Dry milled feed samples were analysed in duplicate for CP, EE, ash, NCGD and NDF using the procedures provided by Association of Official Analytical Chemist (AOAC, 2016) as described in sections 2.1.3, 2.1.4, 2.1.2, and 2.1.5, respectively.

Feed mineral concentration was determined after digestion of dried milled samples with concentrated HNO_3 and HCl, using the DigiPREP digestion system (QMX Laboratories Ltd., Essex, UK) and analysed by ICP-MS after diluting in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK) as described by Cope *et al*. (2009) (see section 2.6). Dry liver samples were analysed following digestion overnight at 60°C in concentrated HNO_3 (70%; Fisher Scientific, UK). Plasma minerals were analysed after dilution (1:20) in 0.5% HNO_3 as described in Section 2.8. The TCA-soluble Cu was analysed as described by Mackenzie *et al*. (1997) (see Section 2.9).

Experiment 2:

Eight Texel cross store lambs, 4 intact and 4 wethers, with a mean LW of 28.4 ± 3.7 kg, were used in a Latin square design experiment to investigate the effect of dietary supplemental Fe doses on apparent absorption of minerals present in the diets used in experiment 1. Lambs were sheared prior to the start of the study then blocked by LW and sex, and randomly assigned to one of four dietary treatments (two lambs per treatment). The experiment consisted of 4 periods and each period ended after 21 days (Table 3.5).

The lambs were treated with anticoccicidial oral suspension (Vecoxan®) at rate of 10 ml per head (Elanco Animal Health, Hertfordshire, UK) prior to the start of study. Throughout the experiment period, lambs were housed indoors individually, in slatted floor pens,

under continuous lighting in a temperature maintained room (15°C). Total faeces and urine were collected during the last 7 days of each 21 days feeding period. Between each change of the experimental diet, lambs were fed the new diet for 14 days to minimize dietary carryover effects. Faecal materials were collected in polyethylene bags and weighed, then a sample of 10% of the daily output was stored at -20°C for further analysis. The faecal sub-samples were mixed thoroughly and then freeze dried before being ground for further analyses. Faecal minerals were determined after ashing and acid digestion as described in section 2.5.

Table 3.5. Diet allocation of eight Texel cross lambs.

Period	Lamb			
	3 + 4	6 + 7	1 + 8	2 + 5
1	H	M	L	C
2	L	C	H	M
3	C	H	M	L
4	M	L	C	H

Diet was offered to the lambs at 1.10x maintenance (AFRC, 1993) feed ingredients used in experiment 2 were similar to those used in experiment 1 (Table 3.2). The chemical and mineral composition of the basal diet used in experiment 2 is present in Table 3.6.

Table 3.6. Chemical composition of the basal diet of experiment 2

Item	Treatments			
	C[1]	L[2]	M[3]	H[4]
DM (g/kg fresh)	904.9	911.5	910.3	905
CP (g/kg DM)	177.9	183.7	174.7	187.3
EE (g/kg DM)	29.5	27.3	31.8	35.8
NDF (g/kg DM)	361.3	363.8	367.5	307.9
OM (g/kg DM)	904.5	904.5	904.5	904.5
Ash (g/kg DM)	95.5	92.9	91.1	95.9
GE (MJ/kg DM)	17.64	17.48	17.29	17.67
Mineral concentration (mg/kg DM)				
Cu	11.1	10.7	11.5	11.1
Fe	1087	1054	1223	1677
Mn	116.8	113.6	107.9	123.1
Mo	4.0	4.0	4.0	4.3
S (g/kg)	3.0	3.0	3.4	3.1
Zn	83.5	81.0	79.0	101.0

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Blood samples were collected on day 21 of each period, into vacutainers containing silica for Cp analysis or K_2EDTA for plasma minerals determination (Becton Dickinson Vacutainer Systems, Plymouth, UK) as described in Section 2.5 and 2.7 respectively. Feed and faecal mineral concentrations were determined after digestion of dried milled feed and faecal ash samples with concentrated HNO_3 and HCl, using the same procedure of experiment 1 (see Section 2.6). Urine mineral concentration was determined using the same procedure used for plasma minerals determination (see Section 2.7).

3.2.5. Data analysis

Repeated measure analysis of variance for weekly weights, blood components, plasma minerals, and for Cp, Cp:Pl-Cu ratio and SOD activity were carried out using GenStat 17th edition (VSN Int. Ltd., Hempstead. UK) as an unbalanced design experiment. Dietary Fe level was the main effect. For haematology analysis, week 0 has set as a covariate. Weekly LWG was calculated by regression analysis and analysed using ANOVA. Data of experiment 2 were analysed as a 4 x 4 Latin square design. All statistical analysis was conducted using GenStat 17th edition. Differences between means were determined using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

3.3. Results

Experiment 1:

3.3.1. Intake and performance characteristics

Repeated measure analysis of variance showed an effect of time on weekly LW of lambs (P<0.001). There was no significant effect of time x Fe interaction on weekly LW of lambs. Supplemental Fe had no effect on the weekly LW of lambs (P>0.05) at any time point throughout the study (Figure 3.1). There was also no significant effect of supplemental Fe on DLWG (Figure 3.2), daily DM intake (DMI), or feed conversion ratio (FCR) between treatments (P>0.05) (Table 3.7).

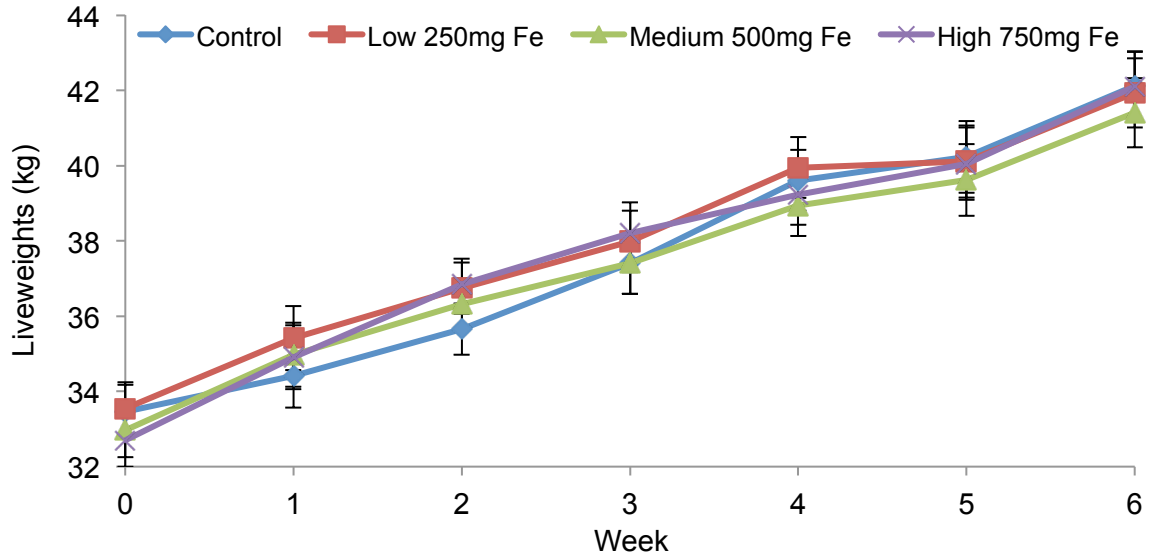

Figure 3.1. Effect of dietary iron on live weight of growing lambs (kg). Error bars are s.e.d.

Table 3.7. Effect of dietary iron on daily live weight gain of growing lambs (kg)

Item	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
Initial wt.	33.46	33.54	32.96	32.70	0.710	0.58
Final wt.	42.12	41.93	41.40	42.10	0.917	0.84
DLWG (kg/d)	0.21	0.21	0.19	0.19	0.018	0.53
DMI (kg/day)	1.13	1.15	1.12	1.13	0.018	0.86
FCR	5.53	6.01	5.93	5.12	0.654	0.42

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

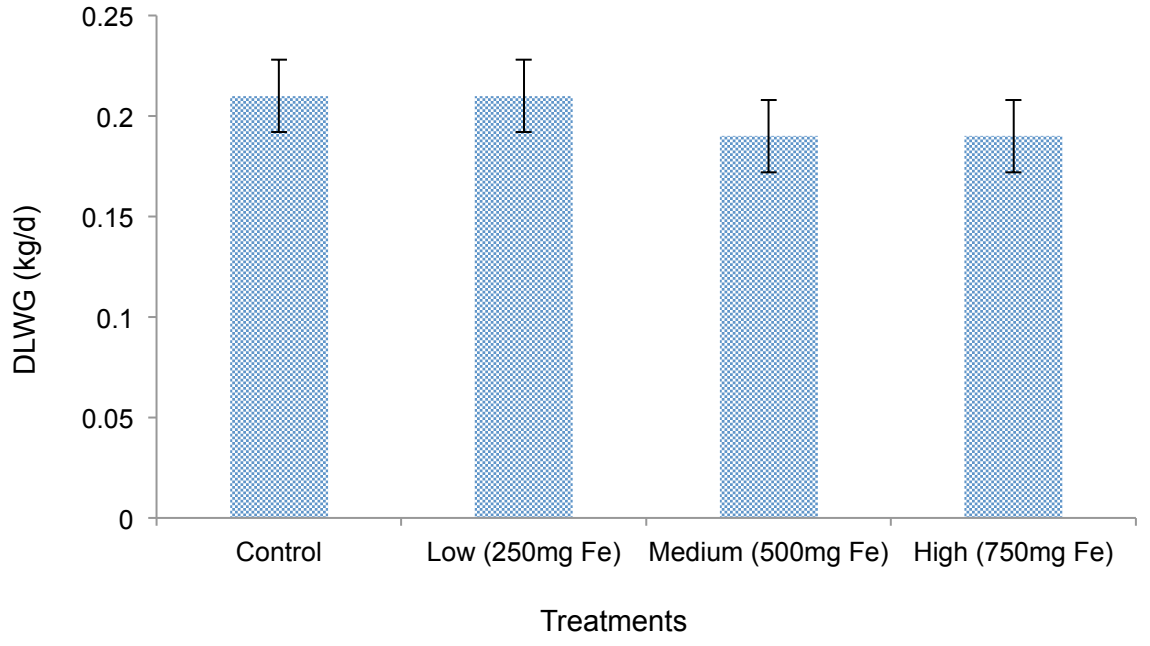

Figure 3.2. Effect of dietary iron on daily live weight gain of growing lambs (kg). Error bars are s.e.d.

3.3.2. Liver mineral concentration

The lambs receiving the H and M diets tended (*P* =0.06) to have a lower liver Cu concentration compared with the lambs fed no Fe supplements or L level of Fe (Table 3.8). The lambs given M or H level of dietary Fe had significantly higher liver Fe concentration compared with lambs fed C or L diets (Figure 3.3). There was no significant effect of supplemental Fe on the liver Mn, Mo, and Zn concentration of lambs after 6 weeks.

Table 3.8. Effect of dietary iron on hepatic minerals concentration of growing lambs (mg/kg DM)

Minerals	Treatment				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
Cu	313.3	322.3	242.8	205.0	49.25	0.06
Fe	362.3[b]	407.0[b]	551.0[a]	613.9[a]	37.04	<0.001
Mn	12.9	11.6	12.7	12.8	1.45	0.79
Mo	1.8	1.6	1.5	1.5	0.24	0.68
Zn	118.0	113.4	122.4	92.2	14.51	0.20

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

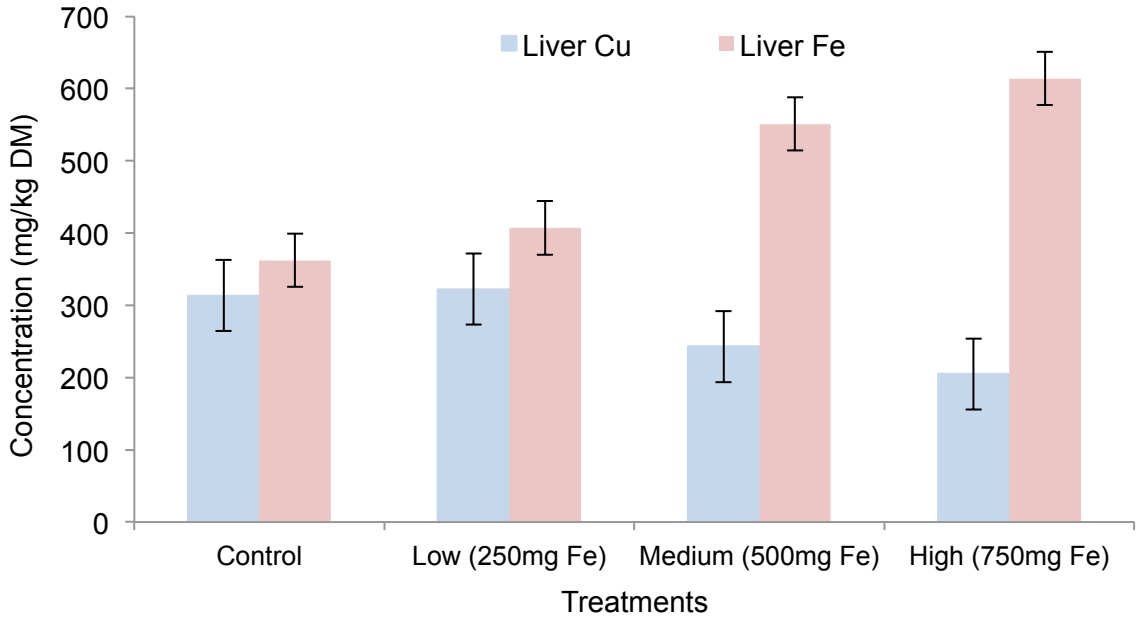

Figure 3.3. Effect of dietary iron on liver copper and iron concentration of growing lambs (mg/kg DM). Error bars are s.e.d.

The lambs given C or L levels of supplemental Fe had a higher (*P*<0.05) total hepatic Cu content compared with those given H or M levels of Fe but there was no significant differences between the lambs given L and M level of Fe (Table 3.9). The total liver Fe content was higher (*P*<0.001) in lambs given M or H diets compared with those given C or

L diets. There was no significant difference in total liver Fe concentration between the lambs given C and L diets or between the lambs given M and H diets. Supplemental Fe had no effect on the total liver Mo, Mn, and Zn concentration.

Table 3.9. Effect of dietary iron on the total hepatic mineral storage of growing lambs (mg/liver)

Mineral	Treatment				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
Cu	54.34[a]	52.45[ab]	36.93[bc]	33.12[c]	7.509	0.02
Fe	62.23[a]	66.87[a]	90.81[b]	100.08[b]	6.092	<0.001
Mn	2.25	1.93	2.10	2.17	0.259	0.66
Mo	0.30	0.25	0.25	0.26	0.043	0.60
Zn	20.15	18.35	20.19	15.22	2.161	0.11

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (P<0.05).

3.3.3. Liver mineral balance

There was no significant effect of supplemental Fe on the liver Cu, Mn, Mo, or Zn retention (Table 3.10). The lambs given M or H levels of Fe retained higher liver Fe (P<0.001) compared with that given C or L diets. There was no significant difference between the lambs given C or L diets (Figure 3.4). Dietary Fe supplements had no effect on liver Mo, Mn or Zn retention.

Table 3.10. Effect of dietary iron on liver mineral balance of growing lambs (µg/day)

Mineral	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
Cu	-0.017	0.154	-1.702	-2.652	1.7430	0.10
Fe	0.520[b]	1.627[b]	4.995[a]	6.542[a]	0.9090	<.001
Mn	-0.050	-0.088	-0.053	-0.058	0.0353	0.81
Mo	0.010	0.003	0.004	0.003	0.0055	0.67
Zn	0.250	0.180	0.365	-0.323	0.3613	0.25

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (P<0.05).

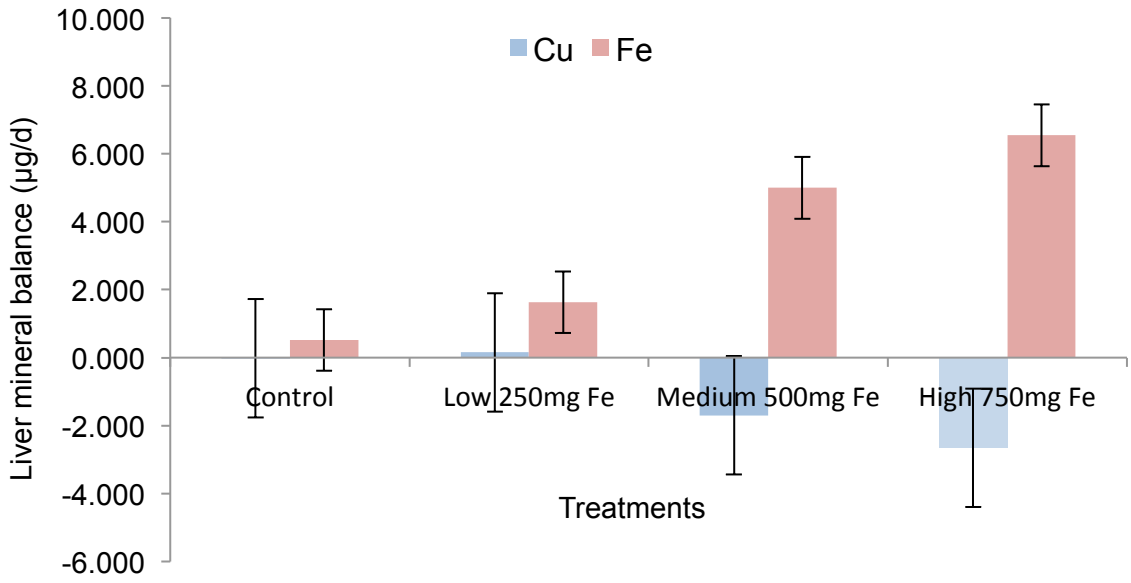

Figure 3.4. Effect of dietary iron on liver copper and iron balance of growing lambs (µg/d) Error bars are s.e.d.

3.3.4. Kidney mineral concentration

There were no significant differences in the kidney mineral concentration between the lambs given different diets by the end of the study (Table 3.11). However, the lambs that received H diet tended to have a lower kidney Cu concentration (*P*= 0.09) compared with those given any of the other diets.

Table 3.11. Effect of dietary iron on kidney mineral concentration of growing lambs (mg/kg DM)

Mineral	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
Cu	10.03	11.31	11.35	8.59	1.192	0.09
Fe	380.6	540.8	528.6	497.1	154.90	0.72
Mn	4.87	6.49	5.99	5.08	0.945	0.28
Mo	2.92	3.45	2.70	2.58	0.393	0.15
Zn	34.08	28.43	45.63	22.16	9.309	0.12

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

3.3.5. Plasma minerals

3.3.5.1. Plasma copper concentration

Repeated measure analysis of variance showed an effect of time (*P*<0.001) on plasma Cu concentration that was decreased with time in all groups. There was no significant Fe x time interaction on plasma Cu concentration of lambs (Table 3.12). There was no significant effect of supplemental Fe on plasma Cu concentration of lambs at any weekly time point throughout the study.

Table 3.12. Effect of dietary iron on plasma copper concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	12.87	13.28	14.30	14.48	1.448	0.62
1	10.36	10.51	11.46	11.10	0.725	0.40
2	10.04	9.93	10.56	10.14	0.854	0.89
3	10.59	10.46	11.37	10.30	0.932	0.68
4	10.93	11.28	12.23	11.90	0.872	0.43
6	11.59	11.50	11.45	10.50	0.589	0.24

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measure analysis:

	P-value
Time	<0.001
Time x Fe	0.39

3.3.5.2. Trichloroacetic acid soluble copper (TCA- soluble Cu)

Repeated measures analysis of variance showed an effect of time (*P*<0.001) on TCA-soluble Cu concentration which decreased by the end of the study. There was no significant time x Fe interaction on TCA- soluble Cu. There was no significant effect of supplemental Fe on TCA- soluble Cu throughout the study (Table 3.13). However, at week 6, the lambs receiving H diet tended to have a lower (*P* =0.09) TCA- soluble Cu compared with those given other diets.

Table 3.13. Effect of dietary iron on plasma TCA- soluble Cu concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	12.59	13.46	14.49	14.28	1.193	0.39
1	11.05	11.17	13.46	11.13	0.297	1.43
2	10.33	11.19	11.36	10.42	0.875	0.54
3	10.89	11.16	12.50	10.96	0.844	0.22
4	9.85	8.98	11.10	10.03	0.965	0.21
6	11.18	10.31	10.35	9.49	0.606	0.09

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measure analysis:

	P-value
Time	<0.001
Time x Fe	0.19

3.3.5.3. Iron concentration

Repeated measures analysis of variance showed a tendency (*P* =0.08) of the effect of time but not time x Fe interaction on plasma Fe concentration. There was no significant effect of supplemental Fe on plasma Fe concentration from week 0 to week 3 (Table 3.14). At week 4 the lambs that received M or H diets had a higher plasma Fe concentration compared with the lambs given C or L diets (*P*<0.05). At week 6 the lambs that received L, M or H had a higher (*P*<0.05) plasma Fe concentration compared with lambs given no Fe supplemental diet. However, there was no significant difference in plasma Fe concentration between lambs given supplemental Fe.

Table 3.14. Effect of dietary iron on plasma iron concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	33.62	46.07	32.39	43.54	7.766	0.25
1	36.16	35.04	45.29	48.08	6.184	0.10
2	39.75	40.72	41.95	40.15	3.752	0.94
3	35.71	44.09	39.84	43.92	6.471	0.55
4	30.85[b]	32.30[b]	36.98[a]	39.61[a]	2.907	0.02
6	28.93[b]	35.93[a]	36.81[a]	39.25[a]	3.344	0.03

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

Repeated measure analysis:

	P-value
Time	0.08
Time x Fe	0.32

3.3.5.4. Manganese concentration

Repeated measure analysis of variance showed a significant effect of time (*P*<0.001) on plasma Mn concentration that was decreased by the end of the study, but there was no significant time x Fe interaction on plasma Mn concentration. Supplemental Fe had no effects (*P*>0.05) on plasma Mn concentration at any weekly time point throughout the study (Table 3.15).

Table 3.15. Effect of dietary iron on plasma manganese concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	0.06	0.05	0.06	0.07	0.012	0.47
1	0.03	0.05	0.05	0.07	0.017	0.22
2	0.09	0.04	0.07	0.05	0.045	0.70
3	0.10	0.09	0.07	0.10	0.011	0.11
4	0.04	0.04	0.05	0.04	0.006	0.15
6	0.04	0.05	0.05	0.04	0.009	0.44

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measure analysis:

	P-value
Time	<0.001
Time x Fe	0.43

3.3.5.5. Molybdenum concentration

Repeated measures analysis of variance showed an effect of time (*P*<0.001), that was declined with time, but not time x Fe interaction on plasma Mo concentrations. Supplemental Fe had no significant effect on the plasma Mo concentration from week 0 to week 3 (Table 3.16). In week 4, the lambs given H diet had a higher (*P*<0.05) plasma Mo concentrations compared to those given all other diets. However, there was no significant difference in plasma Mo concentration between lambs given L and C diets or between the lambs receiving M or C diets. A similar trend was also observed in week 6 (*P* =0.07).

Table 3.16. Effect of dietary iron on plasma molybdenum concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	0.16	0.16	0.13	0.14	0.016	0.19
1	0.10	0.13	0.10	0.11	0.018	0.43
2	0.09	0.11	0.10	0.12	0.019	0.45
3	0.10	0.09	0.08	0.10	0.011	0.11
4	0.10[bc]	0.11[b]	0.08[c]	0.13[a]	0.012	0.002
6	0.11	0.12	0.09	0.14	0.016	0.07

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (P<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.10

3.3.5.6. Zinc concentration

Repeated measures analysis of variance showed an effect of time (P<0.001), that was unclear, but not time x Fe interaction on plasma Zn concentration. Supplemental Fe had no significant effects on plasma Zn concentration at any weekly time point throughout the study (Table 3.17). However, at week 2 the lambs that received H tended (P =0.08) to have a lower plasma Zn concentration than lambs given C.

Table 3.17. Effect of dietary iron on plasma zinc concentration of growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	11.48	12.77	11.11	12.0	1.294	0.63
1	10.0	9.39	10.03	9.69	0.569	0.68
2	10.92	9.52	10.57	8.96	0.786	0.08
3	11.77	10.14	10.17	9.38	0.985	0.40
4	12.14	11.32	11.39	11.27	0.564	0.40
6	11.48	11.03	11.57	11.15	0.797	0.90

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.24

3.3.6. Ceruloplasmin activity (Cp)

Repeated measures analysis of variance showed an effect of time (*P*<0.001), but not time x treatment interaction on plasma Cp activity of lambs. Ceruloplasmin activity was declined with the progress of the study in all lambs groups. There was also no significant effect of supplemental Fe on Cp activity at any weekly time point throughout the study (Table 3.18). At week 3, the lambs given M or H diets tended to have a higher Cp activity (*P* =0.06) compared with lambs fed the control or L diets.

Table 3.18. Effect of dietary iron on ceruloplasmin activity (mg/dl) of growing lambs

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	15.31	17.50	16.69	17.52	1.953	0.68
1	13.76	14.92	15.49	17.09	1.918	0.35
2	12.07	13.44	15.36	14.88	1.610	0.19
3	12.49	13.32	15.49	15.00	1.169	0.06
4	10.25	11.63	12.59	12.24	1.221	0.27
6	12.57	13.28	13.95	12.27	1.416	0.63

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.40

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

Repeated measures analysis of variance showed an effect of time (*P*<0.001), that was unclear, but not time x treatment interaction on Cp:Pl-Cu ratio. The lambs that received supplemental Fe tended (*P* =0.05) to have a higher Cp:Pl-Cu ratio compared with lambs that received no Fe supplements at week 2 (Table 3.19). At week 3, the lambs fed H diet had a higher (*P*<0.05) Cp:Pl-Cu ratio compared with the lambs given C or L diets. However, there was no significant difference between the lambs fed H and M diets, between those given M and L diet or the lambs fed C and L diets in week 3.

Table 3.19. Effect of dietary iron on ceruloplasmin to plasma copper ratio (Cp:Pl-Cu) of growing lambs

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	1.18	1.31	1.18	1.20	0.084	0.38
1	1.34	1.42	1.32	1.53	0.140	0.43
2	1.20	1.35	1.45	1.45	0.094	0.05
3	1.16[c]	1.27[bc]	1.35[ab]	1.51[a]	0.089	0.01
4	0.93	1.03	1.04	1.04	0.085	0.54
6	1.08	1.15	1.22	1.15	0.111	0.70

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).
[a,b] Means with the same superscript are not significantly different (P<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.28

3.3.8. Superoxide dismutase activity (SOD)

Repeated measures analysis of variance showed a significant effect of time but not time x treatment on SOD activity. Superoxide dismutase activity was decreased by the end of the study in all lambs groups. Supplemental Fe had no effect on SOD activity across all treatments at any weekly time point throughout the study (Table 3.20).

Table 3.20. Effect of dietary iron on superoxide dismutase activity (USOD/g Hb) of growing lambs

week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	2266	2030	2110	2178	187.7	0.60
1	1691	1766	1724	1824	213.4	0.97
2	1671	1797	1551	1563	183.1	0.53
3	2033	1959	1835	1915	113.4	0.38
4	1708	1685	1469	1386	225.3	0.41
6	2078	2008	2050	1934	82.9	0.27

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.64

3.3.9. Blood components

3.3.9.1. Red (RBCs) and white (WBCs) blood cells

Repeated measures analysis of variance showed an effect (P<0.05) of time, but not time x Fe interaction on RBC count where RBC increased by the end of the trial in all groups. There was also no significant effect of supplemental Fe on RBC count at any weekly time point throughout the study (Table 3.21). At week 4, the lambs receiving L or H diets had a higher RBC count compared with those given C or M diets (P =0.06). Repeated measures analysis of variance showed no effect (P>0.05) of time or time x Fe interaction on WBC count. Due to the differences in blood components (WBC, Hb, and Hct) between treatments, week 0 was used as a covariate for all blood components ANOVAs. There was no significant effect of Fe supplementation on WBC count at any weekly time point throughout the study (Table 3.22).

Table 3.21. Effect of dietary iron on red blood cell count (x10^9/l) of growing lambs

Week	Treatments				s.e.d.	*P*-value
	C[1]	L[2]	M[3]	H[4]		
0	12.34	12.01	11.49	12.21	-	-
1	12.56	12.31	12.14	12.13	0.339	0.57
2	11.98	12.48	12.81	12.36	0.416	0.33
3	11.15	12.44	12.47	11.87	0.591	0.13
4	11.52	12.31	12.19	11.71	0.324	0.06
6	12.85	13.42	13.06	12.89	0.390	0.29

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	0.04
Time x Fe	0.25

Table 3.22. Effect of dietary iron on white blood cell count (x10^9/l) of growing lambs

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	11.19	11.41	11.59	14.77	-	-
1	11.88	10.14	11.58	12.03	0.886	0.13
2	11.42	11.22	12.73	11.17	0.998	0.33
3	11.24	11.12	12.80	10.81	1.122	0.28
4	11.78	10.84	12.35	10.94	1.254	0.58
6	11.11	11.18	12.46	10.68	1.144	0.44

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	0.88
Time x Fe	0.29

3.3.9.2. Haematocrit percentage (Hct)

Repeated measures analysis of variance showed an effect of time (P<0.05), that was unclear, but not time x treatment interaction on Hct%. There was no significant effect of Fe supplementation on Hct% at any weekly time point throughout the study (Table 3.23).

Table 3.23. Effect of dietary iron on haematocrit percentage of growing lambs

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	33.95	32.22	34.18	33.88	-	-
1	35.29	33.80	34.25	35.29	1.093	0.43
2	34.06	35.00	34.88	34.72	1.104	0.83
3	31.66	33.45	33.30	34.46	1.637	0.40
4	33.01	33.86	33.30	34.36	1.038	0.59
6	32.60	33.14	33.09	34.09	1.104	0.58

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	0.003
Time x Fe	0.53

3.3.6.3. Haemoglobin (Hb)

Repeated measures analysis of variance showed no significant effect of time or time x treatment interaction on Hb concentration. There was also no effect of Fe supplements on Hb concentration at any weekly time point throughout the study (Table 3.24).

Table 3.24. Effect of dietary iron on haemoglobin concentration of growing lambs (g/dl)

Week	Treatments				s.e.d.	*P-value*
	C[1]	L[2]	M[3]	H[4]		
0	12.05	11.52	12.30	12.41	-	-
1	13.34	12.93	13.08	13.14	0.304	0.59
2	12.98	13.37	13.28	13.12	0.467	0.83
3	12.44	13.33	13.00	13.11	0.615	0.51
4	12.50	12.87	12.69	13.10	0.386	0.44
6	12.90	13.28	12.78	13.32	0.362	0.36

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM).

Repeated measures analysis:

	P-value
Time	0.20
Time x Fe	0.49

Experiment 2:

3.3.10. Effect of iron on nutrient and minerals digestibility

There was a significant effect of dietary treatment (*P*<0.05) that was also quadratic (*P* =0.003) no DM, CP, OM, and GE digestibility of lambs (Table 3.25). Similar trend were also found for DE and ME of lambs, but the effect was liner effect of Fe level on NDF digestibility which was reduced with Fe inclusion dose.

Table 3.25. Effect of supplemental iron on nutrient digestibility of growing lambs

Item	Treatments				s.e.d.	*P-value*		
	C[1]	L[2]	M[3]	H[4]		L[5]	Q[6]	C[7]
DM	0.73[a]	0.72[b]	0.71[b]	0.73[a]	0.006	0.55	0.003	0.48
CP	0.74[b]	0.74[b]	0.73[a]	0.75[a]	0.005	0.18	0.003	0.005
NDF	0.65[a]	0.62[b]	0.62[b]	0.60[c]	0.011	<0.001	0.64	0.14
OM	0.75[a]	0.73[b]	0.73[b]	0.75[a]	0.006	0.32	0.002	0.80
Ash	0.53[a]	0.53[a]	0.49[b]	0.52[a]	0.015	0.15	0.11	0.02
GE	0.75[a]	0.73[b]	0.73[b]	0.76[a]	0.006	0.20	<0.001	0.40
Energy values (MJ/kg DM)								
DE	12.8[a]	12.4[b]	12.2[b]	13.0[a]	0.11	0.26	<0.001	0.05
ME	10.35[a]	10.0[b]	9.9[b]	10.5[a]	0.09	0.26	<0.001	0.05

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM). [5] Linear, [6] Quadratic, and [7] Cubic
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

3.3.11. Effect of supplemental iron on copper, manganese and molybdenum absorption and retention

Supplemental Fe had no effect (*P*>0.05) on faecal Cu, Mn, and Mo or on the urinary Cu and Mn excretion of lambs (Table 3.26). Supplemental Fe had also no effect (*P*>0.05) on Cu and Mn retention and apparent absorption of lambs. However, there was a significant linear effect (*P*<0.001) of supplemental Fe on urinary Mo excretion of lambs which was higher with inclusion dose. There was also a linear effect (*P*<0.001) of supplemental Fe on Mo retention and apparent absorption of lambs that was higher in lambs fed no Fe supplemental diet compared with those fed any of the other diets.

Table 3.26. Effects of supplemental iron on Cu, Mn, and Mo excretion (mg/d), retention (mg/d), and apparent absorption of growing lambs

ID	Treatments				s.e.d.	*P-value*		
	C[1]	L[2]	M[3]	H[4]		L[5]	Q[6]	C[7]
Copper								
Intake (mg/d)	4.8	5.0	4.8	4.9	0.12	0.63	0.55	0.22
Faecal excretion	4.9	5.3	4.8	5.1	0.27	0.94	0.60	0.06
Urinary excretion	0.03	0.03	0.03	0.03	0.007	0.50	0.59	0.89
Retention	-0.10	-0.39	0.0	-0.19	0.309	0.91	0.83	0.22
Apparent absorption	-0.025	-0.078	-0.004	-0.039	0.0647	0.89	0.84	0.26
Manganese								
Intake (mg/d)	49.7	51.5	50.2	50.8	1.25	0.62	0.55	0.22
Faecal excretion	24.3	24.9	23.4	25.2	1.07	0.73	0.45	0.13
Urinary excretion	0.103	0.104	0.104	0.097	0.0114	0.62	0.60	0.87
Retention	25.36	26.50	26.67	25.56	1.165	0.84	0.19	0.93
Apparent absorption	0.51	0.52	0.53	0.50	0.018	0.91	0.17	0.36
Molybdenum								
Intake (mg/d)	1.65	1.71	1.67	1.69	0.042	0.62	0.55	0.22
Faecal excretion	0.59	0.60	0.55	0.58	0.030	0.39	0.60	0.13
Urinary excretion	0.56[c]	0.65[bc]	0.70[ab]	0.80[a]	0.053	<0.001	0.93	0.63
Retention	0.50[a]	0.46[a]	0.41[ab]	0.31[b]	0.052	0.001	0.39	0.70
Apparent absorption	0.31[a]	0.27[a]	0.25[a]	0.18[b]	0.029	<0.001	0.50	0.47

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM). [5] Linear, [6] Quadratic, and [7] Cubic

[a,b] Means with the same superscript are not significantly different (*P*<0.05).

3.3.12. Plasma mineral

There was no effect (*P*>0.05) of dietary supplemental Fe no plasma Cu, Fe, Mn, and Zn concentration of lambs (Table 3.27). However, there was a significant linear effect of supplemental Fe on Mo concentration of lambs that was higher (*P*<0.05) in the lambs fed Fe supplemental diets compared with those fed no Fe supplements.

Table 3.27. Effect of supplemental iron on plasma mineral concentration of growing lambs (µmol/l)

Mineral	Treatments				s.e.d.	*P-value*		
	C[1]	L[2]	M[3]	H[4]		L[5]	Q[6]	C[7]
Cu	10.81	12.05	10.88	11.11	0.788	0.92	0.38	0.14
Fe	57.4	56.7	59.8	64.0	4.14	0.10	0.41	0.85
Mn	0.47	0.45	0.44	0.48	0.086	0.88	0.61	0.86
Mo	0.39[b]	0.42[b]	0.49[ab]	0.58[a]	0.066	0.01	0.53	0.95
Zn	10.79	10.22	9.96	10.26	0.514	0.27	0.25	0.88

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM). [5] Linear, [6] Quadratic, and [7] Cubic
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

3.3.13. Ceruloplasmin activity and ceruloplasmin:plasma Cu ratio

Dietary Fe had no effect (*P*>0.05) on Cp activity or Cp:Pl-Cu ratio of lambs (Table 3.28). however, there was a tendency for the quadratic effect of dietary Fe on Cp:Pl-Cu ratio which was lower in lambs fed L or M diets compared with those given C or H diet.

Table 3.28. Effect of supplemental iron on ceruloplasmin (mg/dl) and ceruloplasmin:plasma-copper ratio of growing lambs

ID	Treatments				s.e.d.	*P-value*		
	C[1]	L[2]	M[3]	H[4]		L[5]	Q[6]	C[7]
Cp	12.15	12.68	11.63	13.79	1.387	0.39	0.42	0.29
Cp:Pl-Cu	1.20	1.07	1.08	1.25	0.108	0.62	0.07	0.96

[1]Control diet, [2]Low (250 mg Fe/kg DM), [3]Medium (500 mg Fe/kg DM) and [4]High (750 mg Fe/kg DM). [5] Linear, [6] Quadratic, and [7] Cubic

3.4. Discussion

The mean Fe and Cu content of the basal diet were higher than the predicted values cited by MAFF (1992), especially the Fe content of the basal diet used in experiment 2 (1086.6 mg/kg DM). However, these concentrations were within the ranges observed in ruminant diets (NRC, 1996; Kerr *et al.*, 2008). In the current experiments, supplemental Fe had no effects on performance attributes of lambs. In experiment 2, supplemental Fe had a significant effect on the apparent digestibility of DM, CP, NDF, OM, ash, and GE of lambs. This significant effects does not have important biological values as the lambs were fed similar basal diet and also in experiment 1 no effect was found on animal performance parameters. Prabowo *et al*. (1988) and Williams (2004) also did not find any effects of dietary Fe supplementation (1600 and 500 mg Fe/kg DM, respectively) on sheep performance. These results are also in agreement with the finding of Koong *et al*. (1970) in steer calves fed a diet supplemented with different Fe levels (0, 500, 1000, 2000 mg/kg, as Ferric citrate) for 70 days, and with the results of Humphries *et al*. (1983) and Phillippo *et al.* (1987b) in heifers given a diet supplemented with 800 or 500 mg Fe/kg DM, respectively. However, in another study by Koong *et al.* (1970) when the dietary Fe was increased from 100 to 1000, 2500, or 4000 mg/kg DM, a significant reduction in the DLWG were found with increasing Fe inclusion dose in calves (1.27, 1.19, 0.85, and 0.65 kg/d, respectively). The authors linked the reduction in the DLWG to decreases in the daily feed intake of calves. Koong *et al.* (1970) also suggested that the maximum level of dietary Fe in which the calf can consume safely without a reduction in the amount of feed intake or body gain is approximately 2000 mg/kg DM. In the current study the maximal level of the Fe was much lower (only about 1400 mg/kg DM, in H diet) which could be the reason behind not observing any effects on DM intake of the lambs. Standish *et al*. (1969; 1971) also found a significant reduction in the DLWG of steer calves fed a diet supplemented with 1600 mg Fe/kg DM due to the decrease in the amount of the daily feed intake. In mature sheep, Rosa *et al.* (1986) found a significant decreases in the DLWG when fed a diet supplemented with 1000 mg Fe/kg DM, as ferric citrate, although no significant effects on daily feed intake were observed. This decreases observed in the DLWG could be due to the effect of age of sheep used in the Rosa *et al.* (1986) study. In addition, the feeding regime used in the current study was a restricted feeding unlike the mature sheep used in Rosa *et al.* (1986) study which were fed *ad libitum*.

Animals normally obtain Fe from three main sources: feedstuff, water, and soil ingestion (Campbell *et al*., 1974). For normal physiological body functioning, sheep and cows require 30 - 50 mg Fe/kg DM according to the recommendations of NRC (1980). High dietary Fe in the sheep feed, may have originated from soil contamination, and has been linked to swayback (Suttle, 2010). Sheep ingest soil during grazing, which has been

reported to constitute about 10 - 14% of their daily DM intake (Suttle *et al.*, 1975) or up to 25% in particular situations (Healy, 1974). The maximum tolerable Fe level set for sheep and cattle is 500 mg/kg DM, and most of the diets supplied enough or above the requirements of sheep and cattle and few cases of Fe deficiency have been observed in ruminants (NRC, 2005). The dietary level of 500 mg Fe/kg DM was suggested by ARC (1980) when high levels of dietary Fe were found to reduced DM intake of cattle. In the current studies, the background Fe levels of the diet was at/above the maximum tolerable level set by NRC (2005). However, no significant reduction was observed on lambs performance parameters. This could have been due to species differences or that sheep may have developed a better mechanism than cattle to resist high dietary levels of dietary Fe as sheep often ingest high quantities of soil rich in Fe while grazing.

Dietary Fe tended to decrease liver Cu storage of the lambs given M or H level of supplemental Fe (500 or 750 mg Fe/kg DM, respectively), and significantly reduced the total hepatic Cu storage of lambs supplemented with 750 mg Fe/kg DM. Williams (2004) found a significant reduction in the liver Cu concentration of the lambs given a diet supplemented with 500 mg Fe/kg DM after 10 weeks. Similarly, Prabowo *et al*. (1988) found a significant reduction in liver Cu concentration of lambs given ration supplemented with 1600 mg Fe/kg DM after 98 or 121 days. This significant reduction has been observed by Williams (2004) and Prabowo *et al*. (1988), but the lack of an effect in the current study could have been due to the short period of the study and the levels of supplemental Fe used. Suttle *et al*. (1984) reported an impairment of Cu repletion of the sheep, irrespective of dietary S concentration, when given a diet supplemented with 800 mg Fe/kg DM, as $FeSO_4$ for 21 days.

The reduction in the liver Cu concentration observed in the current study may have been due to reducing Cu availability in the digestive tract as suggested by Phillippo *et al*. (1987a) and Gould and Kendall (2011). However, results of experiment 2 have not shown a reduction in the apparent Cu absorption and retention. It has proposed that Fe^{2+} reacts with S^{2-} in the rumen to form an FeS compound then under the low pH of abomasum ferrous sulphide complex dissociate and sulphide (S^{2-}) may react with Cu to form an insoluble Cu-S complex (Suttle *et al*., 1984). It is also believed that ionic Fe and Cu may compete for the same luminal binding proteins necessary for mucosal transport and when the Fe is high binding preferentially bound to Fe than to Cu (Ashmead and Ashmead, 2004).

In a Latin square design experiment utilising beef steers, a significant reduction in the Cu absorption from the digestive tract was observed when fed a diet supplemented with 1000 mg Fe/kg DM (Standish *et al*., 1971). Humphries *et al*. (1983) and Yu *et al*. (1994) have indicated two ways in which Fe could have the most dramatic effect on Cu metabolism, a)

at the site of absorption in the small intestine, b) increases in the Cu efflux from the liver through bile excretion. High dietary Fe in sheep (Suttle and Peter, 1985) and rats (Yu *et al*., 1994) has decreased apparent Cu absorption. Fry (2011) found approximately 2-fold increase in the mRNA expression of the hepatic Cu efflux pump (ATP7b) in calves fed high Fe (750 mg/kg DM, supplemental Fe). The hepatic Cu exporter (ATP7b) is responsible for the biliary Cu excretion and incorporation of Cu into Cp (Linder, 2002; Kim *et al.*, 2008). The current study measured faecal Cu concentrations to determine apparent absorption only. Therefore, if there was in increase in the expression of ATP7b in the lambs in this study similar to that reported by Fry (2011) we would not be able to differentiate it from a reduction in absorption.

In the current experiments dietary Fe level had no effect on the plasma Cu concentration of lambs. However, a decreasing trend was observed in the lambs given 750 mg Fe/kg DM in week 6. This may have been due to the short period of the study and high liver Cu concentration of lambs by week 6. 90 – 95% of plasma Cu is reported to be associated with Cp which is secreted by the liver (Terada *et al*., 1995). Herdt and Hoff (2011) reported that significant differences in the plasma Cu is difficult to observe when the concentration in the plasma is low. Significant reductions in the plasma Cu concentration can be observed when liver Cu concentration drops to < 25 mg/kg DW (Claypool *et al*., 1975; Mulryan and Mason, 1992). No clinical sign of Cu deficiency was detected throughout both experiments due to the high level of liver Cu by the end of the study. Clinical signs of Cu deficiency can be observed when the liver Cu concentration drop to less than 20 mg/kg DM which is set to be the threshold value for Cu deficiency as estimated by NRC (2007), or to less than 10 mg/kg DM (Suttle, 2010). High levels of dietary Mo are found to cause clinical sign of Cu deficiency, due to molybdenosis as it has similar clinical signs of clinical Cu deficiency (Ward, 1978; Gould and Kendall, 2011). Other researchers have, found a significant reduction in both plasma and hepatic Cu concentration of cattle given diets high in Fe (Standish *et al*. 1971;Campbell *et al*., 1974; Phillippo *et al.* 1987b; 1987a; Bremner *et al*., 1987). However, Bremner *et al*. (1987) did not observe a significant effect of high supplemental Fe on the hepatic Cu concentration of pre-weaned calves, although the effect was significant in weaned calves. Bremner *et al*. (1987) emphasised the important role of the rumen microorganisms in reducing S to sulphide (S^{2-}) that have been suggested to react with Fe first, and then with Cu in the digestive tract to reduce Cu availability (Gould and Kendell, 2011).

The increase in the liver and plasma Fe concentration in a dose response order reflects the presence of the different concentration of Fe in the diets in an available form. This results are in agreement with those previously reported by other researchers in sheep (Prabowo *et al*. 1988; Williams, 2004) and cattle (Standish *et al.,* 1971; Campbell *et al*.,

1974; Humphries *et al*., 1983; Phillippo *et al*., 1978a, 1978b; Bremner *et al*., 1987; Hansen *et al*., 2010). In the current study, increasing liver Fe concentration was followed by the reduction in the liver Cu concentration. Similar results was also observed by Williams (2004) in sheep fed a diet supplemented with 500 mg Fe/kg DM. In cattle, Hansen *et al.* (2010) have also observed that liver Cu tended to be lower, while liver Fe was higher in the calves fed high dietary Fe (supplemented with 750 mg Fe/kg DM) compared to their control counterparts. Standish *et al*. (1971) observed a significant reduction in the concentration of liver Cu, Zn and Mn (and kidney Mn) of steers fed a diet containing a total Fe concentration of 1000 mg/kg DM after 11 weeks. DMT1 has been reported to have an important role in Fe absorption as well as other minerals including Cu absorption from the small intestine (Hansen, 2008; Suttle, 2010). Hansen *et al.* (2010) observed a reduction in the duodenal expression of DMT1 of calves given a diet supplemented with 750 mg Fe/kg DM. Supplemental Fe in experiment 2 had no effects on Cu or Mn absorption and retention but Mo absorption was lower in lambs given 500 mg Fe/kg DM. Standish *et al.* (1971) observed significant reductions in Cu absorption of calves fed a diet supplemented with 1000 mg Fe/kg DM. Dietary Fe had no effect on plasma TCA-soluble Cu in the current trial, which suggests that there was no TM produced in the rumen of lambs or absorbed into the blood (Gould and Kendall, 2011).

In experiment 2, there was a positive effect of supplemental Fe on urinary Mo excretion in a dose response order, and decreases in dietary Mo absorption of the lambs. This finding can be explained by an increase in tissue Mo depletion of the lambs and increases in the plasma Mo concentration that was excreted in the urine as a result of high Fe intakes. However, no mechanisms have been reported to address this increase in the level of urinary and plasma Mo increase. Increase in plasma Mo concentration was also observed previously by Humphries *et al*. (1983) in cattle fed a diet supplemented with 800 mg Fe/kg DM but this issue was not discussed. The mechanisms by which Fe and S reduce liver and plasma Cu concentration is not fully understood. Two hypotheses have been proposed which explain the effect of high dietary Fe on Cu metabolism in ruminants: a-) Fe reacts with S in the rumen and then with Cu in the small intestine producing unavailable FeCuS complexes. b-) Fe reacts with S to produce FeS complexes in the rumen, and then S is substituted with Cu to form FeCu complexes, which is not available for absorption (Gould and Kendall, 2011). The Fe derived from soil contamination is reported to be less available for absorption (Hansen and Spears, 2009). Therefore, ingestion of soil during grazing may have minor effects on the Fe bioavailability of the animals (Hansen and Spears, 2009). Hansen and Spears (2009) suggested that Fe derived from soil contamination of silages during ensiling process represents a good source of available Fe that is readily available for absorption. On the other hand, Suttle *et al*. (1984) have observed a significant reduction in the Cu replete of Scottish Blackface

ewes fed diets supplemented with 100 g/kg DM soil providing either 2.8 and 1.5 g Fe/kg DM when dietary S content of the diet was high. Hansen and Spears, (2009) reported that the increase in the Fe bioavailability derived from soil in the ensiled contaminated forages was suggested to be due to the low silage pH during fermentation.

The increase observed in the plasma Mo concentration of the lambs given M or H diets in the both studies may have been due to Fe increasing Mo depletion from body tissues or Fe may have an effect on Mo absorption from the digestive tract. Similar findings was also observed by Humphries *et al.* (1983) in heifer calves given a diet supplemented with 800 mg Fe/kg DM. The tendency observed for Fe to reduce plasma Zn concentration might have been due to the competition between Fe and Zn absorption from the apical membrane of the enterocytes as both of these minerals share the same transporter protein DMT1 (Aggett, 2012).

In experiment 1, there was a significant effect of time on the plasma mineral concentration of lambs which reduced by the end of the study. This could have been due to the effect of the age of the lambs. The lambs were approximately 3 months old when they were used in the study and young lambs have been reported to be more efficient at absorbing minerals and as they grow the rumen develops and mineral availability reduces due to the effects of microorganism present in the rumen. Bremner *et al*., (1987) found no effect of Fe and S supplementation on Cu status of milk fed calves but after rumen development similar levels of supplemental antagonists had an effect.

Supplemental Fe had no effect on Cp activity of the lambs in both studies, but in week 3 the lambs fed M or H had significantly higher Cp than lambs given no or low Fe. This results disagree with those observed by William (2004) in sheep and other researcher in cattle but agree with those found by Fry (2011) who fed a diet supplemented with 750 mg Fe/kg DM. Similarly, the Cp:Pl-Cu ratio observed in this experiment disagree with the results of Williams (2004) who found a significant reduction in Cp:Pl-Cu ratio of lambs fed a high Fe supplemental diet. The Cp:Pl-Cu ratio has been proposed by Mackenzie *et al*. (1997) to identify Cu deficiency of animals due to TM production. William (2004) found a significant reduction in Cp:Pl-Cu ratio of lambs fed a diet supplemented with 10 mg Mo/kg DM and suggested that this decrease was due to the TM production using the ratios proposed by Mackenzie *et al*. (1997). This increases in the Cp:Pl-Cu ratio of the lambs given high Fe diets needs further investigations to explain the rise in this ratio. Data more relevant to animal nutrition has indicated that Cp mRNA was not affected in rats that were exposed to different levels of dietary Fe supplements (Tran *et al*., 2002). In ruminants the activity of Cp has been found to decrease with Cu depletion during primary Cu deficiency (Blakley and Hamilton, 1985), or after exposure to high levels of dietary Mo intakes (Humphries *et al*., 1983; William, 2004). However, Cp activity may increase as a result of

infection, stress, or pregnancy (Linder, 1991b). Therefore, Cp:Pl-Cu ratio was advocated to be more accurate indicator than plasma Cu or Cp activity alone to assess the functional Cu status of ruminants (Mackenzie *et al.*, 1997). The theoretical ration of 2:1 (Cp:Pl-Cu) was proposed by Mackenzie *et al*. (1997) for normal ruminants, ratio <2.0 that TM was being absorbed into the blood, ratio of 1.0 - 1.5 may indicate a definite TM problem and <1.0, a serious TM problem. This ratio was confirmed by Mackenzie *et al.* (2001). Kendall *et al*. (2001) reported an increase in the Cp:Pl-Cu ratio in cattle supplemented with Cu. In this studies, Cp:Pl-Cu ratio values was lower than what was expected.

3.5. Conclusion

It has been found that supplementation of 500 and 750 mg Fe/kg DM (total concentration of 935.1 and 1399.8 g/kg DM, respectively) significantly reduced total hepatic Cu concentration of the lambs compared with 0 and 250 mg Fe/kg DM. Level of Fe had no effect on lambs performance or on Cu containing enzymes or blood components over the period of the study. With increasing dietary Fe level, liver Fe concentration increased in accordance with the inclusion dose. No effects of supplemental Fe were found on plasma Cu, but Fe and Mo concentration increased in accordance with the level of the Fe inclusion. Urinary Cu was not affected by Fe supplements but urinary Mo increased in groups fed Fe supplementation. No signs of Cu deficiency were observed in the lambs by the end of the studies. Dietary Fe level had no effects on digestibility of DM, CP, NDF, or other parameters. Dietary Fe supplements showed minor effects on the Cu retention of lambs in the balance study.

CHAPTER 4

Effect of dietary sulphur level with or without iron on copper status of sheep

4.1. Introduction

Ruminants like other species, require S for normal body functions. Sulphur-containing compounds that have a metabolic, structural, and regulatory function are ubiquitous (Goodrich and Garrett, 1986). Sulphur is a critical part of some B-vitamins, amino acids, and other cellular components (Suttle, 2010). However, increased S intake can result in polioencephalomalacia (PEM) and decreases animal performance (Gould, 1998). Sheep and cattle metabolise organic and inorganic S, including methionine and sulphate-S (Goodrich and Garrett, 1986). In the rumen all sources of dietary S are reduced to sulphide as a result of the action of microorganisms (Hale and Garrgus, 1953; Starks *et al*., 1953; Lewis, 1954; Johnson *et al.*, 1971; Callaway *et al.,* 2010). Increasing dietary S, as sulphate or S-amino acids, has been observed to reduce Cu absorption and retention of sheep and cattle, possibly through the formation of insoluble Cu sulphides or Cu thiomolybdates (Dick, 1954; Bird, 1970; Suttle, 1974d).

Currently, the recommended level of dietary S is 1.5 g/kg DM in sheep and cattle feeds (NRC, 2005). The maximum tolerable levels range from 0.3 to 0.5% S for diets containing less than 15% forage and at least 40% forage respectively, to avoid the deleterious effects of elevated dietary S on animal health (Pogge *et al*., 2014). Mineral status of sheep and cattle may be compromised when fed a high S containing diet; high dietary S may antagonise Cu, Se, Zn, Ca, and Mg absorption, availability, and use by the animal (Suttle, 1974; Spears *et al*., 1985; Suttle, 1991; Pogge *et al*., 2014). Thiomolybdates produced from the interaction between S and Mo in the rumen lead to impaired absorption and systemic use of Cu in the body (Kandylis, 1984; Suttle, 1991; Gould and Kendall, 2011).

Substantial amounts of Fe may be obtained through soil ingestion while animals are grazing (Healy, 1970), or soil contamination of harvested forages (Rafferty *et al.*, 1994). Dietary Fe intakes of as low as 250 to 500 mg/kg DM have been linked to Cu deficiency in sheep and cattle (Humphries *et al*., 1983; Bremner *et al.*, 1987; Phillippo *et al.*, 1987a; Prabowo *et al*., 1988; Williams, 2004; Hansen and Spears, 2009; Fry, 2011).

It has been suggested that the antagonistic effects of Fe in ruminants arise from its interaction with sulphide produced in the rumen, this may result in trapping of the sulphide

and enhancement of its ability to restrict Cu absorption in the small intestine (Suttle *et al.*, 1984). This proposition is based partially on the observation that the effects of soil ingestion on Cu availability can be related to the Fe content of the soil and are dependent on adequate dietary S intake in sheep. Under this scenario, sulphide may be able to react with Cu, forming insoluble Cu-sulphide complexes. Phillippo *et al*. (1987a) also proposed a mechanism by which Fe alters Cu metabolism in cattle through reducing Cu absorption by formation of insoluble FeCuS salts in the gut. Yet no clear mechanism responsible for this effect has been elucidated.

The Suttle and McLauchlin (1976) predicted Cu availability based on the concentration of Mo + S of the diet but had no consideration for the level of Fe in the diet. Therefore, regression analysis of the data reported in Chapter 3 and the data obtained from Williams (2004) work were conducted on the effect of dietary Fe on the hepatic Cu retention and the equation was obtained to use in the current study to reduce liver Cu retention by 50% (Figure 4.1).

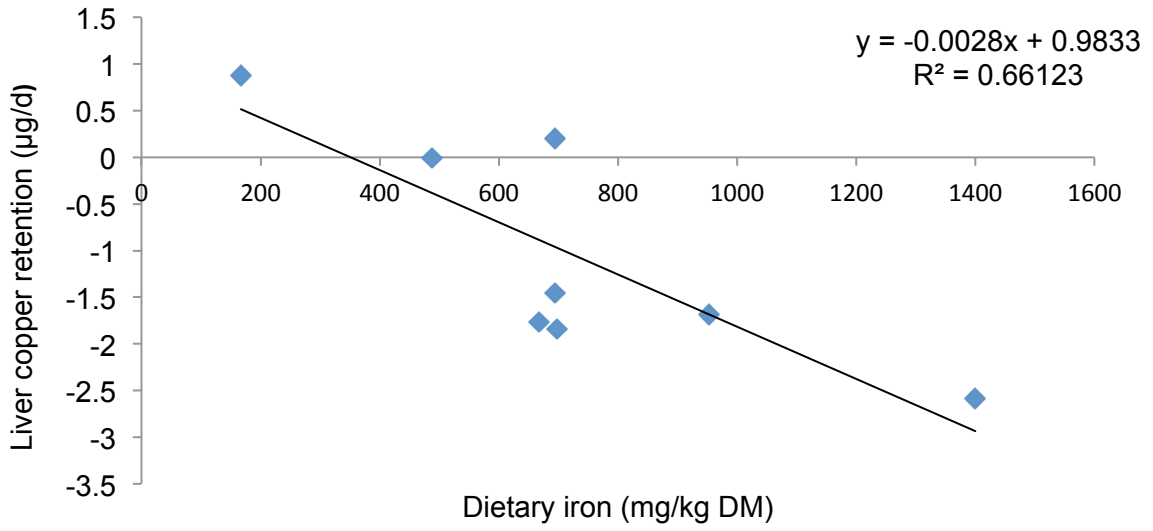

Figure 4.1. Correlation between the total dietary iron (mg/kg DM) and liver copper retention (µg/d) of growing lambs (Data from Section 3.3.3. and Williams, 2004)

However, it does include antagonistic effect of S on Cu metabolism of sheep. Therefore, the aim of this study was to determine the effects of different levels of dietary S with or without Fe on Cu metabolism and performance of growing lambs.

4.2. Materials and Methods

All procedures were conducted in accordance to the UK Animals (Scientific Procedures) Act 1986 and approved by the Harper Adams University Research Ethics Committee before initiation of the trial.

4.2.1. Animals and experimental design

Sixty-two Texel crossbred weaned lambs (31 castrates and 31 females) with an average LW of 29.1 ±3.7 kg were used in a 2 × 3 factorial design experiment. The lambs used in this experiment were obtained from Harper Adams University flock, which were all grazed at pasture prior to the start of this study. The lambs were blocked by LW and sex and then 8 random lambs were selected and slaughtered in a commercial abattoir by stunning and exsanguination prior the initiation of the trial to determine the initial liver minerals concentration of lambs (Table 4.1). The remaining fifty-four lambs were randomly assigned to one of six dietary treatments and fed for 13 weeks (see section 4.2.2). The lambs were housed individually in a metal pen in a naturally ventilated barn, and bedded with wood shaving for the duration of the experiment.

Table 4.1. Liver minerals concentration of the lamb slaughtered on day 0 (mg/kg DM) (n=8, ±SD)

Mn	Fe	Cu	Zn	Mo
20.1 ±6.48	392.5 ±77.34	591.1 ±281.23	94.5 ±7.92	2.6 ±1.01
(29.1, 20.9)[a]	(524.7, 306.3)	(945.6, 237.0)	(103.2, 84.2)	(4.4, 1.3)

[a](Maximum value, Minimum value)

4.2.2. Diet formulation

The raw feed ingredients used in the current experiment were chosen on the basis of published (MAFF, 1992) and analysed low Cu content. The diets were mixed on farm using a Hi Spec Mix Max Super 10 feeder wagon (Hi Spec, Carlow, ROI) after calibration to +/- 1kg. The basal diet was formulated to supply 10.24 MJ/kg DM metabolisable energy (ME) and 86.17 g/kg DM metabolisable protein (MP), and rationed for lambs weighing 30 kg to grow at rate of 200 g per day (AFRC, 1993). The basal diet was predicted to supply 95.55 g/kg ERDP and 8.92 MJ/kg FME. Lambs were assigned to one of six dietary treatments as presented in Table 4.2.

Table 4.2. Experimental treatments

	Dietary treatments					
	No Iron			Iron (800 mg/kg DM)		
Sulphur (g/kg DM)	0	1.5	3.5	0	1.5	3.5
T. Code	L:L	L:M	L:H	H:L	H:M	H:H

L= low, M= medium, and H= high

The raw material and chemical composition of the basal diet are presented in Table 4.3.

The experiment had six treatments consisting of:

Treatment 1**:** basal ration (L:L)

Treatment 2**:** basal diet supplemented with 1.5 g S/kg DM (L:M)

Treatment 3**:** basal diet supplemented with 3.5 g S/kg DM (L:H)

Treatment 4**:** basal diet supplemented with 800 mg Fe/kg DM (H:L)

Treatment 5**:** basal diet supplemented with 800 mg Fe/kg DM and 1.5 g S/kg DM (H:M)

Treatment 6**:** basal diet supplemented with 800 mg Fe/kg DM and 3.5 g S/kg DM (H:M).

Supplemental S was added as reagent grade ammonium sulphate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA) and Fe as iron sulphate $FeSO_4.7H_2O$ (Fisher Scientific., Leicester, UK). The S supplements supplied by the $FeSO_4.7H_2O$ was balanced with $(NH_4)_2SO_4$ accordingly prior to mixing the diets. All diets were balanced for N using feed grade urea (Trouw Nutrition, Northwich, Cheshire, UK).

Table 4.3. Diet formulation (g/kg) and chemical composition of the experimental diets

Item	Treatments					
	L:L	L:M	L:H	H:L	H:M	H:H
Nutritionally improved straw (NIS)	400	400	400	400	400	400
Barley	202	202	202	202	202	202
Sugar beet pulp	150	150	150	150	150	150
Soya bean meal	150	150	150	150	150	150
Molasses	50	50	50	50	50	50
Megalac	20	20	20	20	20	20
Urea	3	3	3	3	3	3
Mins/vits premix[1]	25	25	25	25	25	25
Chemical composition (g/kg DM)						
DM	877.8	877.0	875.2	875.2	877.3	878.8
CP	16.4	17.1	15.2	18.0	17.5	18.9
NDF	456.7	449.4	430.4	462.8	508.9	482.8
EE	21.5	20.3	20.9	20.0	20.36	20.6
Ash	100.6	103.3	93.6	97.0	100.9	99.4
OM	899.4	896.7	906.4	903.0	899.1	900.6
Mineral concentration (mg/kg DM)						
Cu	9.5	11.6	8.7	9.6	10.2	11.4
Fe	302	285	296	1400	1402	1402
Mn	72.4	74.0	73.7	74.0	76.0	75.4
Mo	1.2	1.5	1.6	1.8	1.7	1.9
S (g/kg DM)	2.9	3.3	3.6	3.6	4.3	5.5
Zn	78.7	83.7	62.3	86.9	95.9	94.9

[1] Mineral premix (25 kg/ ton) (RUMENCO LTD., Burton upon Trent, UK) containing 320,000 IU/kg Vit A, 100,000 IU/kg Vit D3, 2,000 IU/kg Vit E, 18.5% calcium, 2.0% phosphorous, 1.0% magnesium, 12.0% sodium, 25% chloride20 mg/kg selenium, 90 mg/kg cobalt, 150 mg/kg iodine, 3000 mg/kg manganese, and 3000 mg/kg zinc.

4.2.3. Experimental routine

Lambs were monitored daily and feed was rationed to support a predicted live LWG of 200 g/d (AFRC, 1993). Feed was offered individually in to clean plastic buckets to avoid mineral contamination. Feed samples (about 1 kg) were collected weekly and stored at -20°C for the subsequent chemical analyses. Water was available *ad-libitum*. Feed refusals were recorded twice weekly.

4.2.3.1. Live weight determination

The LW of the lambs was recorded weekly on Monday at 11:00 h using a balance (Shearwell Data Ltd., Somerset, UK). Metric standard weights were used to calibrate the scale prior to weighing (F.L. Thornton and Co. Ltd., Wolverhampton, UK). The weekly LW of lambs was used to calculate daily feed offered the following week to achieve DLWG of 200 g/d (AFRC, 1993). The DLWG was calculated by regression analysis based on weekly weights.

4.2.3.2. Blood sampling and analysis

Jugular blood samples were obtained on Tuesdays at 11:00 h in weeks 0, 2, 4, 6, 8, 10, and 12 for serum and plasma and in weeks 0, 4, 8, and 12 for whole blood. Blood was also collected into vacutainer tubes containing K_2EDTA designed for determining plasma minerals concentration (Cu, Fe, Mn, Mo, and Zn). Plasma was collection by centrifugation at 1000 ×g for 10 min at 4°C (Sigma Laboratory Centrifuges., Germany). Plasma was removed and stored at -20°C for subsequent analysis as described in section 2.2. Whole blood samples, collected in Vacutainer spray coated with K_2EDTA, was analysed for WBC, RBC, Hct and Hb using Vet Haematology Analyser device (Woodley Equipment Co Ltd., Bolton, UK) as described in section 2.3. Subsamples of whole blood were also taken and stored at -20°C for the subsequent SOD activity determination using Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in section 2.4. Vacuum tubes for serum collection (silica coated) were stored overnight at 4°C before being centrifuged at 1000 ×g for 10 min at 4°C and serum was stored at -20°C for subsequent analyses. Serum was used for determining Cp activity based on the method of Henry *et al*. (1974), using a Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in section 2.5.

4.2.4. Tissue sample collection and analytical procedures

At the end of week 13, all lambs were transported to a commercial abattoir and slaughtered by stunning and exsanguination. Whole livers and gallbladders were collected immediately after slaughter. Livers weight were recorded and samples were taken (~35 g) and stored at -20°C for further analysis. Bile was collected and stored at -20°C for subsequent analysis.

Weekly concentrate samples were bulked and oven dried (Binder, Cole-Palmers, UK) at 105°C overnight (see section 2.1.1). Dry feed samples were milled using a Delonghi KG79

grinder (Freemans PLC, Sheffield, UK) and analysed in duplicates for CP, EE, ash, and NDF according to Association of Official Analytical Chemist (AOAC, 2016) as described in section 2.1.3, 2.1.4, 2.1.2, and 2.1.5 respectively.

Feed mineral concentration were determined after digestion of dried milled samples with concentrated HNO_3 and HCl, using DigiPREP digestion system (QMX Laboratories Ltd., Essex, UK) and analysed by ICP-MS after diluted in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK) as described by Cope *et al*. (2009) (section 2.6). Dry liver samples were analysed following digestion overnight at 60°C in concentrated HNO_3 (70%; Fisher Scientific., UK). Plasma and bile samples were analysed after dilution (1:20) in 0.5% HNO_3 (section 2.8). Liver and hay reference samples were used to monitor consistency and reliability of specific minerals concentration (European Commission, Rerieseweg, Belgium). Trichloroacetic acid (TCA) soluble Cu fraction of plasma was obtained via separating the supernatant after precipitation of plasma proteins with 10% TCA solution (Sigma-Aldrich Co., Germany). The TCA supernatant was analysed by ICP-MS as described in section 2.9.

4.2.5. Statistical analysis

Weekly live weights, blood components, plasma minerals, Cp, Cp:Pl-Cu ratio and SOD activity were analysed by repeated measures analysis of variance with week 0 as a covariate for live weights, SOD activity and haematology data using GenStat 17th edition (VSN Int. Ltd., Hempstead. UK). All data were analysed by a two way ANOVA as a 2 × 3 factorial design, with the main effects being Fe and S. DLWG was determined by regression analysis and analysed by ANOVA. Differences between means were determined using protected least significant difference (LSD) (Snedecor and Cochran, 1989).

4.3. Results

4.3.1. Intake and performance characteristics

Repeated measure analysis showed a significant effect of time (P<0.001) on the weekly LW, DMI and FCR of lambs, but there was no effect of time x Fe, time x S, or time x Fe x S interaction on weekly LW, DMI, or FCR of the lambs.

There was no significant effect of Fe, S or Fe x S on weekly LW, DLWG, DMI or FCR of lambs at any weekly time point throughout the study (Table 4.4). However, at week 10 the lambs fed diets supplemented with Fe trended (P =0.08) to have a lower LW compared with lambs receiving no Fe supplementation (mean 38.42 vs 39.43 kg, respectively) (Figure 4.2). There was also a trend (P = 0.09) for the effect of Fe x S interaction on the daily DM intake (DMI) by the end of the study which was slightly lower in lambs fed H:H diet compared with those given any of the other diets.

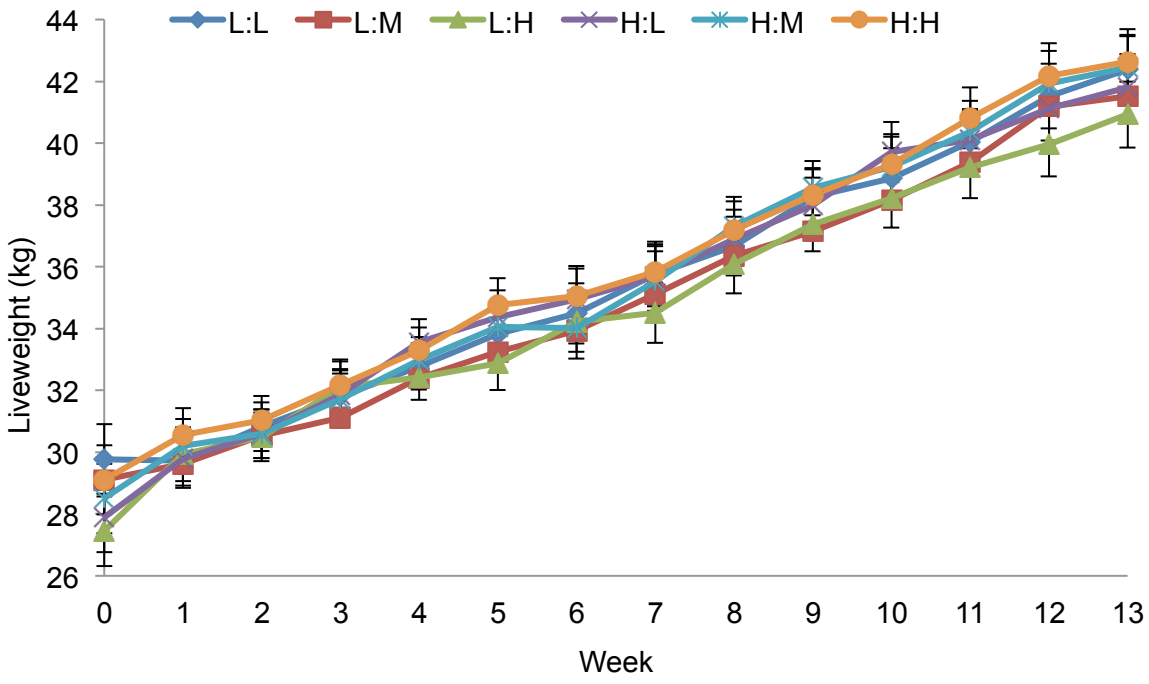

Figure 4.2. Effect of sulphur with or without iron on weekly live weight of lambs (kg). Error bars are s.e.d.

Table 4.4 Effect of sulphur with or without iron on intake and performance of growing lambs (kg)

Item	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
Initial wt.	27.9	28.5	29.1	29.8	29.1	27.4	-	-	-	-
Final wt.	41.8	42.4	42.6	42.4	41.5	40.9	1.06	0.26	0.93	0.35
DLWG (kg/d)	0.15	0.15	0.15	0.15	0.14	0.13	0.010	0.21	0.64	0.35
DMI (kg/day)	1.08	1.10	1.12	1.13	1.09	1.07	0.030	0.85	0.85	0.09
FCR (kg/kg)	7.4	7.4	7.2	7.6	7.7	7.5	0.68	0.50	0.95	0.97

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

4.3.2. Liver mineral concentration

There was no significant Fe x S interaction on liver Fe, Mn, Mo, and Zn concentration (Table 4.5). However, there was a trend (*P* =0.06) for Fe x S interaction on liver Cu concentration by the end of the study. Liver Cu concentration was significantly lower in lambs that received Fe supplemental diets compared those given no Fe supplemental diets (304 vs. 439 mg/kg DM, s.e.d. =44.1, respectively). The lambs that received supplemental Fe had significantly higher liver Fe concentration compared with lamb fed no Fe supplemental diets (Figure 4.3). Dietary Fe had no effects on liver Mn or Mo concentration by the end of the study. The lambs that received high Fe diets tended (*P* =0.06) to have higher liver Zn concentration compared with lambs given no supplemented Fe (143.0 vs. 134.4 mg/kg DM, s.e.d. =4.49, respectively). There was no significant effect of supplemental S on the liver mineral concentration.

Table 4.5. Effect of sulphur with or without iron on liver minerals concentration of growing lambs (mg/kg DM)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
Cu	545	344	427	274	332	306	76.5	0.004	0.42	0.06
Fe	355	320	355	889	817	928	83.7	<.001	0.45	0.81
Mn	23.8	27.1	25.8	29.0	28.0	23.2	6.22	0.75	0.79	0.68
Mo	3.91	3.56	4.08	3.93	3.83	3.83	0.342	0.95	0.52	0.57
Zn	138.3	128.3	136.7	148.3	139.2	141.6	7.77	0.06	0.23	0.84

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

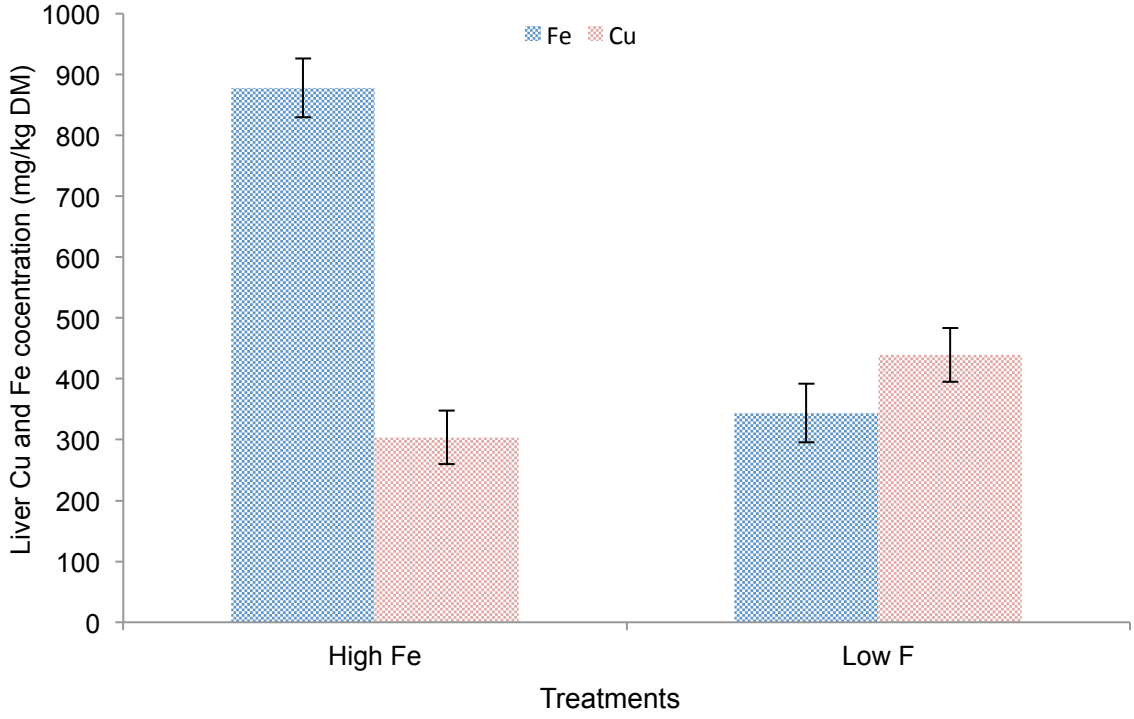

Figure 4.3. Effect of supplemental iron on liver iron and copper concentration of growing lambs (mg/kg DM). Error bars are s.e.d.

There was no significant Fe x S on the total liver mineral storage by the end of the study. The lambs that received no supplemental Fe had significantly higher total liver Cu concentration (*P* =0.005) compared with those given supplemental Fe (77.3 vs. 53.1 mg/liver, s.e.d. =8.29, respectively). The lambs that received supplemental Fe had a significantly higher total hepatic Fe compared with those given no Fe supplements (Table 4.6). There was no significant effect of S supplemental diet on the total liver Cu, Fe, Mn, Mo, and Zn content by the end of the study.

Table 4.6. Effect of sulphur with or without iron on the total liver mineral storage of growing lambs (mg/liver)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
Cu	93.70	62.20	75.80	49.60	56.50	53.20	14.360	0.005	0.48	0.18
Fe	61.30	57.00	61.70	155.90	138.70	158.50	15.900	<0.001	0.50	0.77
Mn	3.93	4.87	4.36	5.18	4.69	3.88	1.035	0.74	0.66	0.46
Mo	0.67	0.63	0.71	0.70	0.66	0.64	0.066	0.84	0.66	0.52
Zn	23.72	22.75	24.00	26.31	23.87	24.10	2.024	0.28	0.50	0.69

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

4.3.3. Liver mineral balance

There was no significant Fe x S on liver Fe, Mn, Mo, or Zn balance. There was a trend (*P* =0.06) of Fe x S interaction on liver Cu balance by the end of the study (Table 4.7). The lambs given no supplemental Fe retained a significantly higher liver Cu per day compared with lambs receiving Fe supplemental diets (-1.64 vs. -3.09 µg/d, s.e.d =0.475, respectively). The lambs given C diet tended to loss less Cu per day compared with those receiving all of the other diets. The lambs given Fe supplemental diets retained significantly higher liver Fe compared with the lambs given no Fe (5.22 vs. -0.52 µg/d, s.e.d =0.519, respectively) (Figure 4.4). The lambs given supplemental Fe also tended (*P* =0.06) to retain more hepatic Zn per day compared with the lambs given no Fe supplemental diets (5.22 vs. 0.43 µg/d, s.e.d =0.048, respectively). There was no significant effect of supplemental S on liver mineral balance.

Table 4.7. Effect of sulphur with or without iron on liver mineral balance of growing lambs (µg/d)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
Cu	-0.49	-2.66	-1.76	-3.41	-2.78	-3.07	0.822	0.005	0.42	0.06
Fe	-0.40	-0.78	-0.40	5.34	4.56	5.76	0.900	<0.001	0.45	0.81
Mn	0.04	0.08	0.06	0.10	0.09	0.03	0.067	0.75	0.79	0.68
Mo	0.01	0.01	0.02	0.01	0.01	0.01	0.002	0.95	0.52	0.57
Zn	0.47	0.36	0.45	0.58	0.48	0.51	0.083	0.06	0.23	0.84

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

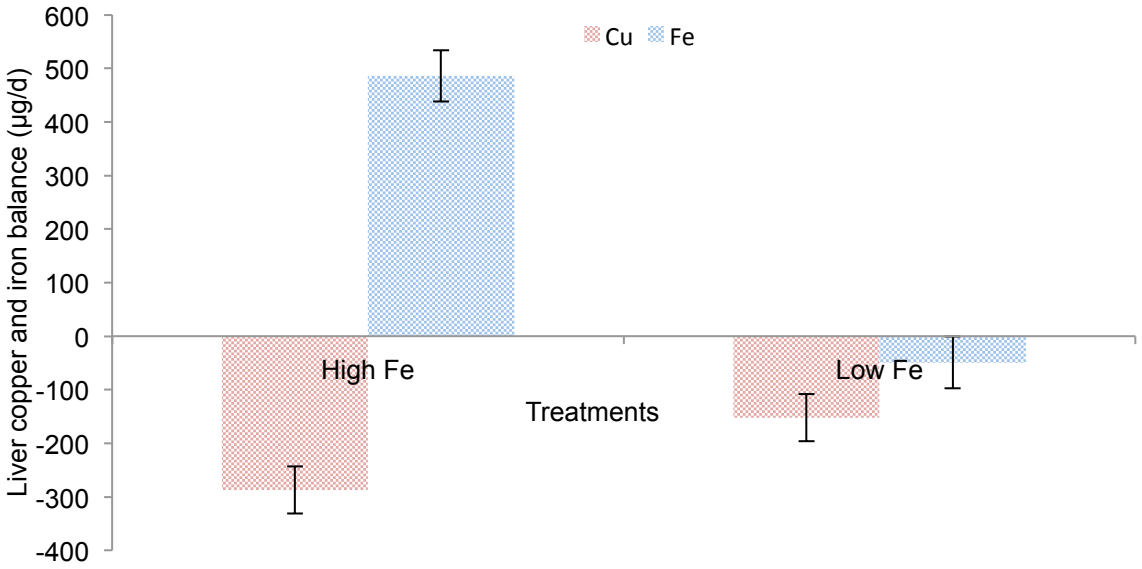

Figure 4.4. Effect of supplemental iron on liver copper and iron balance of growing lambs (µg/d). Error bars are s.e.d.

4.3.4. Biliary mineral concentration

There was a significant Fe x S interaction on biliary Mo concentration, where the lambs receiving H:H diet had a higher biliary Mo concentration than those fed L:H (Table 4.8). There was no significant Fe x S on the biliary Cu, Fe, Mn, and Zn concentration of lambs on week 13. However, the lambs given H:H tended (*P* =0.09) to have a higher biliary Cu concentration compared with those receiving L:H. There was no main effect of supplemental Fe or S on the biliary Cu, Mn, and Fe concentration of lambs at week 13. Biliary Mo concentration was higher (*P* = 0.02) in the lambs that received Fe supplemental diets compared with those given no Fe supplements (0.018 vs. 0.12 µmol/l; s.e.d. = 0.025, respectively). The lambs given Fe supplements tended to have a higher (*P* =0.06) biliary Zn concentrations compared with those given no Fe (34.8 vs. 26.8 µmol/l; s.e.d. = 4.13, respectively). Supplemental S had no effect (*P*>0.05) on the biliary mineral concentration of lambs at week 13.

Table 4.8. Effect of sulphur with or without iron on biliary mineral concentration of growing lambs (µmol/l)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
Cu	5.30	6.16	2.95	4.35	4.82	7.50	2.072	0.53	0.90	0.09
Fe	40.0	54.9	28.7	36.9	38.6	35.2	13.41	0.58	0.31	0.49
Mn	14.34	13.10	13.25	12.89	14.07	11.51	2.533	0.62	0.73	0.71
Mo	0.12^{ab}	0.16^{ab}	0.08^{a}	0.15^{ab}	0.16^{ab}	0.24^{b}	0.044	0.02	0.75	0.004
Zn	28.7	30.3	21.4	38.0	31.4	35.0	7.16	0.06	0.61	0.46

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

4.3.5. Ceruloplasmin activity (Cp)

Repeated measures analysis of variance showed a significant effect of time (*P*<0.001) and time x Fe (*P*<0.05) on Cp activity of lambs. Ceruloplasmin activity was decreased with time by the end of the study in all lambs groups, but to a lesser degree in the groups given supplemental Fe. There was no significant time x S or time x Fe x S interaction on Cp activity of lambs (Table 4.9). There was a significant Fe x S at week 4, where the lambs given H:H had a significantly higher Cp activity compared with those given L:L, L:M, or L:H diets. The lambs given Fe supplemental diets had a higher Cp activity (*P*<0.05) compared with those given no Fe supplements from week 4 to week 12. There was no significant effect of supplemental S on Cp activity from week 0 to week 10. At week 12, the lambs given L:L diet had a higher Cp activity (*P*<0.05) compared with those given L:H.

Table 4.9. Effect of sulphur with or without iron on ceruloplasmin activity of growing lambs (mg/dl)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
0	20.37	19.16	18.14	19.01	17.73	18.36	2.396	0.54	0.66	0.86
2	18.66	15.96	15.87	16.38	16.17	18.58	2.020	0.85	0.56	0.23
4	14.11^{bc}	16.49^{b}	12.04^{c}	16.86^{ab}	17.10^{ab}	19.93^{a}	1.935	0.002	0.63	0.03
6	13.43	16.49	14.26	16.21	16.11	17.82	1.633	0.04	0.40	0.21
8	14.44	15.57	14.06	17.11	18.02	16.49	1.797	0.02	0.49	1.0
10	13.92	12.83	11.14	13.49	15.04	15.06	1.458	0.03	0.71	0.12
12	13.46	11.63	11.16	15.09	15.30	12.50	1.341	0.01	0.04	0.42

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.
[a,b] Means with the same superscript are not significantly different (P<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.01
Time.S	0.36
Time.Fe.S	0.14

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

Repeated measures analysis of variance showed a significant effect of time on Cp:Pl-Cu ratio which was unclear, and of time x Fe that was higher in groups fed supplemental Fe compared to the lambs fed any of the other diets, but no significant time x S or time x Fe x S interaction on Cp:Pl-Cu ratio. There was no significant Fe x S interaction on Cp:Pl-Cu ratio at any weekly time point throughout the study (Table 4.10). The lambs that received Fe supplemental diets had a higher Cp:Pl-Cu ratio in week 4 (P<0.001), 6 (P =0.009), 8 (P =0.001), and 12 (P<0.001) compared with those given no Fe. There was no effect of S supplements on Cp:Pl-Cu ratio at any weekly time points except at week 6, where the lambs given L:L had the lowest (P<0.05) Cp:Pl-Cu ratio than the lambs given L:M or L:H.

Table 4.10. Effect of sulphur with or without iron on ceruloplasmin to plasma copper ratio (Cp:PL-Cu) of growing lambs

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
0	1.17	1.25	1.26	1.15	1.10	1.22	0.123	0.32	0.65	0.74
2	1.20	1.10	0.99	1.18	1.08	1.19	0.088	0.29	0.18	0.13
4	1.10	1.20	1.02	1.20	1.36	1.40	0.093	<0.001	0.19	0.14
6	1.05	1.25	1.19	1.21	1.40	1.33	0.094	0.01	0.004	0.98
8	1.13	1.26	1.16	1.39	1.37	1.42	0.104	0.001	0.73	0.52
10	1.47	1.40	1.21	1.30	1.52	1.68	0.137	0.24	0.84	0.07
12	1.11	0.99	1.00	1.32	1.42	1.20	0.099	<0.001	0.21	0.18

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures:

	P-value
Time	<0.001
Time.Fe	0.01
Time.S	0.35
Time.Fe.S	0.10

4.3.7. Superoxide dismutase activity (SOD)

Repeated measures analysis of variance showed a significant effect of that was unclear, and time x Fe interaction on SOD activity that was higher in the lambs fed no Fe supplemental diets by the end of the study (Table 4.11). There was no significant time x S or time x Fe x S interaction on SOD activity of growing lambs. There was no significant Fe x S interaction on SOD activity at any weekly time point throughout the study. There was also no significant effect of Fe on SOD activity from week 0 to week 8. At week 12, Lambs that received Fe supplements had significantly lower SOD activity compared with those given no Fe supplements (2051.0 vs. 2302.0 USOD/g Hb, s.e.d. =103.1, respectively). There was no significant effect of supplemental S on the SOD activity of lambs at any weekly time point throughout the study.

Table 4.11. Effect of sulphur with or without iron on superoxide dismutase activity of growing lambs (USOD/g Hb)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
0	2005	2123	2123	2319	1972	2018	154.7	-	-	-
4	2235	2088	2272	2398	2389	2292	187.3	0.14	0.84	0.57
8	2505	2464	2377	2377	2589	2615	282.3	0.62	0.93	0.67
12	2351	2328	2228	1943	2141	2071	181.7	<0.05	0.76	0.60

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures:

	P-value
Time	<0.001
Time.Fe	0.04
Time.S	0.56
Time.Fe.S	0.58

4.3.8. Plasma minerals

4.3.8.1. Plasma copper concentration

Repeated measures analysis of variance indicated that there was a significant effect of time on plasma Cu concentration, with it being higher at the start of the study (Table 4.12). There was no significant Fe x time or S x time interaction on plasma Cu concentration. There was a significant time x Fe x S interaction on plasma Cu concentration that was lower by the end of the study compared to other groups. There was a significant Fe x S interaction at week 4 where the lambs that received the H:H had a significantly higher plasma Cu concentrations, while L:H had the lowest Cu concentration. However, there was no significant differences between lambs that received L:L or L:M, or between those given H:L, H:M or H:H diets in week 4.similar trend (*P* =0.05) was observed in week 6.

There was no significant effect of supplemental Fe or S on plasma Cu concentration from week 0 to10. At week 12, the lambs that received Fe supplemental diets had significantly lower plasma Cu concentrations compared with those that given no Fe (10.88 vs. 11.65 µmol/l, s.e.d. =0.436, respectively). The lambs given L:M or L:H diets tended (*P* =0.06) to have a lower plasma Cu concentrations compared with those given L:L diet in week 12.

Table 4.12. Effect of sulphur with or without iron on plasma copper concentration of growing lambs (µmol/l)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	FexS
0	16.67	15.58	14.48	14.80	16.13	15.14	1.321	0.78	0.48	0.32
2	15.64	14.64	15.73	13.65	15.18	15.45	1.393	0.48	0.62	0.43
4	12.72^{ab}	13.52^{ab}	11.68^{b}	13.61^{ab}	12.52^{ab}	14.25^{a}	0.978	0.15	0.96	<0.05
6	12.67	13.15	11.87	13.32	11.64	13.40	0.877	0.66	0.63	0.05
8	12.62	12.44	11.79	12.38	12.78	11.62	0.788	0.96	0.22	0.85
10	9.86	9.58	9.40	10.66	10.60	9.32	1.065	0.35	0.45	0.74
12	12.11	11.67	11.16	11.49	10.78	10.38	0.599	0.03	0.06	0.95

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.
[a,b] Means with the same superscript are not significantly different (P<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.215
Time.S	0.449
Time.Fe.S	0.046

4.3.8.2. Plasma trichloroacetic acid soluble copper (TCA-Cu soluble)

Repeated measures analysis of variance showed that there was a significant effect of time on TCA-Cu soluble concentration, which decreased by the end of the study. There was no significant time x Fe, time x S or time x Fe x S interaction on TCA-Cu soluble concentration. There was no significant Fe x S interaction on TCA-Cu soluble concentration at any weekly time pints throughout the study (Table 4.13). However, at week 6, there was a tendency (P =0.08) for an Fe x S interaction with the lambs receiving L:M having the highest plasma Cu concentration and the lambs given H:M had the lowest. There was no significant effect of dietary Fe or S on the plasma TCA-Cu soluble concentration at any weekly time point throughout the experiment. However, from week 8 to week 12, the lambs given the high level of S tended to have a lower plasma TCA-Cu soluble concentration. The lambs that received supplementary Fe tended (P =0.09) to have a lower plasma TCA-Cu soluble concentration compared with lambs fed no supplemental Fe.

Table 4.13. Effect of sulphur with or without iron on plasma TCA- copper soluble concentration of growing lambs (µmol/l)

Item	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	14.88	14.93	13.31	14.15	14.03	13.37	1.498	0.55	0.46	0.89
2	13.10	12.34	13.13	11.73	12.75	13.03	1.174	0.61	0.70	0.55
4	10.74	11.80	10.59	11.79	11.12	12.41	0.815	0.13	0.91	0.10
6	10.78	12.06	10.56	11.77	9.68	10.42	1.055	0.41	0.58	0.08
8	11.43	11.43	10.47	11.20	11.60	9.82	0.842	0.62	0.06	0.79
10	10.23	10.49	9.64	10.13	10.13	9.18	0.577	0.36	0.07	0.90
12	10.58	10.60	10.06	10.15	10.27	9.13	0.562	0.09	0.08	0.73

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.33
Time.S	0.27
Time.Fe.S	0.12

4.3.8.3. Plasma iron concentration

Repeated measures analysis of variance indicated that there was a significant effect of time on the plasma Fe concentration with levels increasing in all weeks (Table 4.14). There was no significant time x Fe, time x S or time x Fe x S interaction on plasma Fe concentration. There was no significant Fe x S interaction on the plasma Fe concentration of lambs at any time point throughout the study. Plasma Fe concentration was significantly higher in lambs that received Fe supplemented diets in weeks 2 (P<0.05), 4 (P<0.001), 8 (P =0.001), 10 (P =0.006), and 12 (P<0.05) compared with lambs given no Fe. Dietary S had no significant effect on plasma Fe concentration at any weekly time points throughout the study.

Table 4.14. Effect of sulphur with or without iron on plasma Fe concentration of growing lambs (µmol/l)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	37.1	36.0	43.0	58.4	38.9	40.9	12.60	0.32	0.52	0.39
2	27.3	29.4	31.6	39.4	34.0	31.7	3.95	0.02	0.78	0.11
4	42.2	37.9	40.0	47.6	50.0	45.7	3.10	<0.001	0.65	0.23
6	32.1	29.2	50.7	40.3	42.0	54.4	12.36	0.26	0.10	0.88
8	37.1	36.7	36.4	47.8	52.8	48.4	6.33	0.001	0.84	0.82
10	35.7	29.6	30.9	39.1	38.8	41.1	4.51	0.01	0.60	0.53
12	44.3	41.6	44.5	66.4	47.9	53.1	10.32	0.01	0.35	0.51

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.833
Time.S	0.297
Time.Fe.S	0.755

4.3.8.4. Plasma manganese concentration

Repeated measures analysis of variance showed a significant effect of time on plasma Mn concentration with levels increasing with the progress of the study (Table 4.15). There was no significant time x Fe, time x S or time x Fe x S interaction on plasma Mn concentration. There was no significant Fe x S interaction on the plasma Mn concentration of lambs at any weekly time points throughout the study. There was also no significant effect of supplemental Fe on plasma Mn concentration of lambs at any weekly time points throughout the study. At week 6, lambs that received the L:H diet had significantly higher plasma Mn concentration than lambs given L:L or L:M diets, and the lambs given L:L diet had significantly higher plasma Mn concentration than those given L:M diet.

Table 4.15. Effect of sulphur with or without iron on plasma Mn concentration of growing lambs (µmol/l)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	0.37	0.57	0.37	0.41	0.37	0.42	0.153	0.70	0.69	0.44
2	1.00	1.20	0.90	0.65	0.67	1.16	0.326	0.28	0.68	0.21
4	0.52	0.36	0.95	0.35	0.52	0.74	0.203	0.52	<0.01	0.37
6	1.04	0.78	1.01	0.92	0.74	0.90	0.216	0.49	0.30	0.97
8	0.64	0.40	0.34	0.41	0.60	0.33	0.182	0.92	0.29	0.26
10	1.68	1.45	1.18	1.64	1.61	1.15	0.494	0.92	0.35	0.95
12	0.52	0.61	0.51	0.68	0.55	0.50	0.181	0.77	0.74	0.69

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.77
Time.S	0.20
Time.Fe.S	0.61

4.3.8.5. Plasma molybdenum concentration

Repeated measures analysis of variance showed a significant effect of time, with the levels increasing with time, time x Fe (*P*<0.001), which was higher with time in groups fed supplemental Fe, time x Fe x S (*P*<0.001), which was higher with time in groups fed supplemental Fe with S with the progress of the study and time x S (*P* = 0.002) on plasma Mo concentration that increased with time (Table 4.16). At week 2, there was a tendency (*P* =0.06) for an Fe x S interaction on plasma Mo concentration where the lambs given H:H diet having a higher plasma Mo concentration compared with the lambs give the other diets. From week 4 to 12, there was a significant Fe x S where the lambs given Fe with S had a significantly higher plasma Mo concentration than those given all of the other diets. However, there was no significant differences in plasma Mo concentration between the lambs given H:L and H:M or between the lambs given L:L and L:M from week 4 to 12 or L:H in week 8 and 10. From week 2 to 12, the lambs that given supplemental Fe had significantly higher plasma Mo concentration compared with the lambs given any of the other diets. From week 0 to 4, there was no effect of supplemental S on plasma Mo concentration. However, from week 6 to 10 the lambs that received L:M or L:H diet had significantly lower plasma

Mo concentration than the lambs given L:L diet, but the effect was not significant in week 12.

Table 4.16. Effect of sulphur with or without iron on plasma molybdenum concentration of growing lambs (µmol/l)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	0.09	0.09	0.13	0.11	0.09	0.08	0.024	0.7	0.73	0.12
2	0.16	0.14	0.12	0.22	0.22	0.25	0.023	<0.001	0.98	0.06
4	0.19^{c}	0.15^{cd}	0.11^{d}	0.24^{ab}	0.22^{bc}	0.27^{a}	0.021	<0.001	0.1	0.003
6	0.14^{bc}	0.10^{c}	0.09^{c}	0.18^{b}	0.18^{b}	0.33^{a}	0.024	<0.001	<0.001	<0.001
8	0.13^{c}	0.11^{c}	0.09^{c}	0.22^{b}	0.19^{b}	0.29^{a}	0.02	<0.001	0.02	<0.001
10	0.13^{c}	0.10^{c}	0.09^{c}	0.20^{b}	0.20^{b}	0.34^{a}	0.028	<0.001	0.01	<0.001
12	0.17^{c}	0.18^{bc}	0.13^{d}	0.24^{b}	0.22^{b}	0.30^{a}	0.021	<0.001	0.57	<0.001

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

Repeated measures analysis:

	P
Time	<0.001
Time.Fe	<0.001
Time.S	0.002
Time.Fe.S	<0.001

4.3.8.6. Plasma zinc concentration

Repeated measures analysis of variance indicated an effect of time (*P*<0.001) on plasma Zn concentration that was higher by the end of the study. There was a trend (*P* =0.08) for the effect of time x Fe on plasma Zn concentration. There was also a trend (*P* =0.098) for the effect of time x Fe x S on plasma Zn concentration.

There was no significant Fe x S on the plasma Zn concentration at any weekly time points throughout the study (Table 4.17). At week 2 there was a trend (*P* =0.095) for the effect of Fe x S on plasma Zn concentration which was lower in the groups fed H:M and H:H compared with the lambs fed any of the other diets. There was no significant effect of Fe on plasma Zn concentration at any weekly time point throughout the study except at week 4, when the lambs given Fe supplements had a significantly higher plasma Zn concentration compared with lambs given no supplemental Fe (10.39 vs. 9.78 µmol/l, s.e.d. =0.282, respectively). At week 2, there was a tendency (*P*= 0.07) for the lambs

that received L:M or L:H to have a higher plasma Zn concentration compared with the lambs given the all of the other diets. At weeks 4, 6, 8, and 12, the lambs receiving L:M or L:H had a significantly lower plasma Zn concentration compared with those given no supplements. However, there was no significant differences between those given L:M and L:H diets.

Table 4.17. Effect of sulphur with or without iron on plasma Zn concentration of growing lambs (µmol/l)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	8.6	7.94	8.07	8.81	8.29	7.65	0.685	0.91	0.22	0.7
2	10.78	10.26	10.95	11.3	9.56	9.15	0.73	0.13	0.07	0.10
4	10.41	9.38	9.57	11.31	10.31	9.55	0.488	0.04	0.001	0.31
6	11.62	9.66	8.91	10.72	9.37	9.79	0.582	0.76	<0.001	0.10
8	11.37	10.25	10.06	11.73	10.61	10.17	0.548	0.38	0.002	0.93
10	9.67	8.71	8.97	10.35	9.13	9.27	0.935	0.39	0.23	0.96
12	12.37	10.74	10.93	11.95	11.07	10.90	0.614	0.91	0.01	0.69

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	<0.001
Time.Fe	0.08
Time.S	0.69
Time.Fe.S	0.10

4.3.9. Blood components

4.3.9.1. Red (RBCs) and white (WBCs) blood cells

Due to differences between blood components between treatments, week 0 was uses as a covariate for all blood components ANOVAs. Repeated measures analysis of variance showed a significant effect of time on RBC and WBC cells count which was unclear (Table 4.18 and Table 4.19, respectively). However, there was no significant time x Fe, time x S or time x Fe x S interaction on either RBC or WBC count. There was no significant interaction or main effect of Fe or S on RBC or WBC count at any weekly time points throughout the study.

Table 4.18. Effect of sulphur with or without iron on RBCs count of growing lambs (M/mm^3)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	12.91	11.86	11.57	11.67	11.85	12.84	0.669	-	-	-
4	12.39	11.74	11.94	11.44	11.92	11.48	0.612	0.26	0.89	0.43
8	12.07	11.86	11.58	12.06	12.09	12.29	0.48	0.27	0.93	0.56
12	11.55	11.62	11.12	10.72	12.09	11.5	0.651	0.99	0.27	0.31

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	0.02
Time.Fe	0.20
Time.S	0.34
Time.Fe.S	0.85

Table 4.19. Effect of different levels of sulphur with or without iron on WBCs count of growing lambs (M/mm^3)

Week	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	10.22	10.74	10.76	10.19	11.02	9.98	1.060	0.78	0.65	0.77
4	11.05	11.01	11.48	11.46	11.52	10.94	1.296	0.87	1.00	0.82
8	10.88	11.80	11.79	11.89	13.09	10.70	1.233	0.58	0.34	0.34
12	10.21	10.80	12.02	10.81	11.98	10.37	1.392	0.96	0.65	0.33

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	0.01
Time.Fe	0.90
Time.S	0.87
Time.Fe.S	0.90

4.3.9.2. Haematocrit (Hct)

Repeated measures analysis of variance showed a tendency for the effect of time (*P* =0.06), but not time x treatment interaction on blood Hct percentage. There was no

significant Fe x S on Hct percentage at any weekly time points throughout the study (Table 4.20). Haematocrit percentage was significantly higher in lambs that receiving supplemental Fe compared with those given on Fe supplements in week 8, and a tendency (*P* =0.06) in week 12. Dietary supplemented S had no effect (*P*>0.05) on Hct percentage at any time point throughout the study.

Table 4.20. Effect of sulphur with or without iron on haematocrit of growing lambs (%)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	34.16	31.03	30.68	31.54	32.34	35.99	2.248	-	-	-
4	32.57	31.66	32.22	31.41	33.23	30.60	1.620	0.68	0.67	0.32
8	32.46	32.75	31.49	33.66	34.76	34.00	1.216	<0.05	0.46	0.77
12	30.32	31.32	32.25	31.92	35.12	32.54	1.694	0.06	0.11	0.64

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	0.06
Time.Fe	0.09
Time.S	0.50
Time.Fe.S	0.80

4.3.9.3. Haemoglobin (Hb)

Repeated measures analysis of variance showed a significant effect of time, that was lower by the end of the study, and time x Fe interaction, that was higher in groups fed supplemental Fe by the end of the study, on Hb, but there was no significant time x S or time x Fe x S interaction on Hb concentration. There was no significant Fe x S interaction on Hb concentration at any weekly time point throughout the study (Table 4.21). At week 8 and 12, the lambs receiving supplemental Fe had higher Hb concentration compared with those given no supplemental Fe. Dietary S had no significant effect on Hb concentration at any weekly time point throughout they study.

Table 4.21. Effect of sulphur with or without iron on haemoglobin concentration of growing lambs (g/dl)

ID	Treatments						s.e.d.	*P-value*		
	L:L	L:M	L:H	H:L	H:M	H:H		Fe	S	Fe x S
0	14.46	12.96	13.08	12.63	13.77	14.01	0.797	-	-	-
4	12.64	12.40	12.29	12.11	12.47	11.87	0.641	0.43	0.69	0.79
8	11.97	12.38	11.94	12.95	12.97	12.77	0.451	<0.05	0.58	0.84
12	12.04	13.15	12.06	13.44	13.79	13.33	0.769	<0.05	0.28	0.76

L:L= Low Fe:Low S, L:M= Low Fe:Medium S, L:H=Low Fe:High S, H:L=High Fe:Low S, H:M=High Fe:Medium S, and H:H= High Fe:High S.

Repeated measures analysis:

	P-value
Time	0.02
Time.Fe	0.009
Time.S	0.66
Time.Fe.S	0.57

4.4. Discussion

Dietary S supplements with or without Fe did not cause symptoms of hypocuprosis or general ill health in lambs throughout the study which is in agreement with the results reported in Chapter 3 and also with the finding of other researcher in sheep (Prabowo *et al*., 1988; Williams, 2004) and cattle (Humphries *et al*., 1983; Bremner *et al.,* 1987; Phillippo *et al*.; 1987a). The lambs fed supplemental S alone also showed no symptoms of Cu deficency throughout the studywhich is in agreement with the results found by Grace *et al*. (1997, 1998) when sheep were given high doses of S, ranging from 3.9 to 7.9 g/d, for 105 days. Similarly, Pogge *et al*. (2014) also found no effect of S (2.4 and 6.8 g/kg DM, as sodium sulphate) on DMI and DM or OM digestibility of steers.

In contrast, Zinn *et al*. (1997) found a 20% reduction in DMI, 9.03% in DLWG, and 16.25% in FCR in steers given 2.5 g S/kg DM compared with those given 1.5 or 2.0 g S/kg DM . In another study by Spears *et al.* (2011), they found no effect of supplemental S (0, 1, or 3 g/kg DM) on the DMI of steers during the growth stage, but steers given 3 g S/kg DM had a lower DLWG compared with those given 0 or 1 g S/kg DM. These results disagree with those observed in the current study, which could have been due to the species difference or to other unknown dietary effects. Grace *et al*. (1997) cited that excessive S intake might be toxic along with an influence on absorption and storage of Cu. High S intakes of 6.3 g S/kg DM have been reported to cause polioencephalomalacia in sheep (Olkowski *et al*., 1992).

The performance measurements were not influenced by the dietary treatments. Chapter 3 data showed no effects of supplemental Fe on DM and nutrient digestibility, so the energy and protein utilisation will remain the same and no effect will be expected on lambs performance. These results are in accordance with the results found by other researchers in sheep (Prabowo *et al*., 1988; Grace *et al*., 1998; Williams, 2004), and cattle (Coup and Campbell, 1964; Campbell *et al*., 1974; Bremner *et al.,* 1987; Phillippo *et al*., 1987a; Gengelbach *et al*., 1994).

In the current experiment, the lambs given H:L diet had lower liver Cu by 43.85% relative to lambs given the L:L diet. This finding confirms the antagonist effect of Fe on liver Cu storage of lambs similar to the results reported in Chapter 3 and of other researcher in sheep (Prabowo *et al*., 1988; Williams, 2004) and cattle (Bremner *et al*., 1987; Phillippo *et al*. 1987a; Gengelbach *et al*., 1994; Fry, 2011). On the other hand, Rosa *et al*. (1986) found no significant differences in liver Cu stores of sheep given diet supplemented with 1000 mg Fe/kg DM (as ferric citrate), that may have been due to the chemical form of the Fe used in their study as different forms of Fe have a different absorption availability (Fritz *et al*., 1970).

Bremner *et al*. (1987) observed no significant effect of increasing levels dietary Fe (50, 250, and 500 mg/kg DM) on the liver Cu retention of pre-ruminant calves fed a milk substitute after 8 weeks. However, supplemental Fe reduced liver and plasma Cu concentration of weaned calves in all groups supplemented with Fe. Bremner *et al*. (1987) suggested that this inconsistent result could be due to the role of microorganisms in the functional rumen of weaned calves. Microorganisms in the functional rumen have a significant role in reducing S to sulphite that reacts with Mo to produce TM and subsequently binds Cu and reduce its absorption (Gould and Kendall, 2011). The Mo level of the diet was low (average = 1.62 mg/kg DM) which is unlikely to react with S in the rumen to produce TM. This was confirmed by observing no significant differences in the TCA-Cu soluble between treatments. When Cu:Mo ratio is low (<1) then TMs leave the rumen into the general circulation through rumen wall (Price *et al.*, 1987), while in the current study Cu:Mo ratio was >7.5. Grace *et al*. (1997) found that increasing Cu intakes (from 9.3 to 24.3 mg Cu/d) elevated liver Cu concentrations three-fold, regardless of the presence or absence of elemental-S (3.9 to 7.9 g/d). Similarly, in the current study dietary S with and without supplemental Fe had no significant effects on the liver Fe, Mn, Mo, and Zn concentration although there was a tendency for F x S on liver Cu concentration. Previous studies reported that increasing dietary S (as sulphate-S and methionine) has altered Cu absorption and metabolism (when basal Mo is <0.5 mg/kg DM). Suttle (1974d) also found that increasing S intakes as sulphate-S or methionine from 1 to 4 g S/kg DM reduced Cu absorption coefficient of sheep from 0.062 to 0.041.

The lambs fed Fe supplements had 30.75% lower liver Cu concentration compared with those given no supplements. Opposite to the current results, Pogge *et al*. (2014) found a significant reduction in the liver Cu and Zn concentration of steers given a diet high S (6.8 g S/kg DM) compared with those given low S diet (2.4 g/kg DM). Similar reductions in liver and plasma Cu concentration was reported by Spears *et al*. (2011) in steers given a diet supplemented with 1.5 or 3 g S/kg DM. Increasing dietary S intakes, as sulphate or S-amino acids, has been observed to reduce Cu absorption and storage in sheep perhaps through the formation of insoluble Cu sulphides or Cu thiomolybdates (Dick 1954; Bird 1970; Suttle 1974d). Fertilisation of sorghum with 138 kg S/ha (as (NH4) 2SO4) resulted in a 50% decrease in the apparent Cu absorption when the lambs fed ensiled sorghum in a metabolism trial (Ahmad *et al*., 1995). The relationship between the Cu absorption and the dietary S concentrations is curvilinear (Grace *et al*., 1997). Suttle and McLauchlin (1976) found that increasing dietary S concentration from 0.5 to 2.0 g/kg DM causes a greater reduction in the Cu absorption than increasing dietary S from 3 to 5 g/kg DM. Suttle (1974d) has suggested that the possible mechanism to describe the S x Cu interaction is that increased dietary S elevates the concentrations of free S^{2-} in the digestive tract with the subsequent formation of insoluble CuS complex that is excreted in

the faeces. In the current study, level of dietary S had a minimal effect on liver and plasma Cu concentration.

The increases in the liver Fe concentration of the lamb group fed supplemental Fe was in agreement with the results reported in Chapter 3 and with the results reported by other researchers in sheep (Rosa *et al*., 1986; Prabowo *et al*., 1988; Williams, 2004) and cattle (Campbell *et al*., 1974; Phillippo *et al*., 1987a; Bremner *et al*., 1987). The increases in the liver Fe concentration of the lambs given Fe supplements (about 60.82% more) versus those given no supplements, indicated that the Fe form used in the current study was more available for absorption and storage (Ammerman *et al*., 1967).

Both organic and inorganic forms of S have been shown to have an independent effect on Cu metabolism, possibly through the formation of insoluble CuS at sites beyond the rumen, on liver Cu stores when present in a high concentrations in sheep (Wynne and McClymont, 1956; Goodrich and Tillman, 1966; Suttle, 1974d; Suttle and McLauchlin, 1976; Ginocchio *et al*., 2002) and cattle (Hartmans and Grift, 1964; Bremner *et al*., 1987). However, the severity of dietary S effect on liver Cu storage is further enhanced when the dietary Mo concentration was high (Dick and Bull, 1945; Marcilese *et al*., 1969b). Microorganisms present in the functional rumen reduces S to S^{2-} before it reacts with Mo to form different classes of TM that bind with Cu in the rumen and render Cu unavailable (Dick *et al*., 1975). In the case of low ruminal Cu concentration, TM may enter into the general circulation through rumen walls to bind with Cu in biological molecules to produce a systemic effect and reduce the activity of Cu containing enzymes (Gould and Kendall, 2011). The effect of S on Cu availability from a semi-purified diet was measured in an experiment by Suttle (1974c) using plasma Cu as an indicator of the Cu status of Scottish Blackface ewes. An increase in the S content of the low Mo containing (0.5 mg/kg DM) diet from 1 to 4 g/kg DM was found to reduce Cu availability by approximately 40%, the basal diet contained 1.0 - 1.3 g S and 1.5 mg Cu/kg DM. Results of the current experiment disagree with those reported by Suttle (1974c) and supplemental S did not significantly reduce liver or plasma Cu concentration. Suttle *et al*. (1984) reported that S was vital to inhibit Cu absorption in sheep fed diets supplemented with 100 g/kg DM soil rich Fe.

The current finding is contradictory to those published by several researchers who suggested that there is a combined effect of Fe and S on reducing liver Cu concentration of the animal (Suttle *et al*., 1984; Suttle and Peter, 1985; Bremner *et al*., 1987), but was in agreement with those reported by Grace *et al*. (1997) who found no significant effects of increasing dietary S on plasma or liver Cu concentration. Similar results were found in young cattle fed a diet containing 1.8 to 3.3 g S/kg when Mo was >0.5 mg/kg DM; although an increase in the ruminal S^{2-} concentrations were observed (Mills *et al*., 1977). The contradictory results of the effect of S and Fe on liver Cu observed in different studies

could be due to the levels of S and other minerals in the diet that may have interfered with Fe and S effect on Cu absorption and excretion.

The significant reduction of plasma Cu concentration observed at weeks 12 in the lambs given supplemental Fe diets is in agreement with the results reported by other researchers in sheep (Suttle *et al*., 1975; Williams, 2004) and cattle (Standish *et al*., 1969; Humphries *et al*., 1983; Phillippo *et al*., 1987a). Although no significant effect of supplemental Fe was found in Chapter 3 that may have been due to the short period of the study. No additive effect of Fe and S were found to reduce plasma or liver Cu concentration of lambs, in agreement with the results reported by Bremner *et al.* (1987) in pre-ruminant calves given a milk-substitute high in Fe and S. The increases in the plasma Fe concentration found in the current study confirm the highly availability of supplement Fe form which was in agreement with the results reported in Chapter 3 and with the result observed by Bremner *et al.* (1987) in calves fed diets supplemented with Fe. Plasma Mo increased in the groups of lambs given supplemental Fe which is similar with the result observed in Chapter 3 in lambs given diet supplemented with 750 mg Fe/kg DM, and also with the results reported by Humphries *et al*. (1983) in cattle fed 800 mg/kg DM supplemented Fe. Similar to the results observed in Chapter 3, time had a significant effect on plasma minerals of lambs, as concentrations were lower by the end of the study. These can be due to the high concentration of these minerals in the plasma of lambs at the start of the study. Younger ruminants have a higher efficiency of minerals absorption compared to adults due to the action of microorganisms present in the rumen environment (Suttle, 2010).

High dietary Fe may reduce liver and plasma Cu concentration by different mechanisms which could be different from Mo effect and have not yet been specified. The increases in the biliary Mo concentration observed in the current experiment in groups fed diet supplemented with 800 mg Fe and 3 g S/kg DM was parallel with the increases in the plasma Mo concentration of lamb groups given supplemental Fe. The increase in the blood and biliary Mo concentration may have an effect on reducing liver Cu storage of lambs given Fe supplements or a combination of Fe and S diet however, no clear mechanisms have been reported to explain plasma and biliary Mo given supplemental Fe.

Dietary Fe increased Cp activity and Cp:Pl-Cu ratio of lambs which disagrees with the result observed by Williams (2004) in sheep fed a diet supplemented with 500 mg Fe/kg DM or with the results of Campbell *et al.* (1974) and Humphries *et al*. (1983) in cattle given 30 mg Fe/kg live weight and 800 mg Fe/kg DM, respectively. Previous researchers found a significant reduction in Cp activity of animals given a high Fe diet. Fry (2011) found a significant increases in the Cp concentration of weaned calves given diets supplemented with 750 mg Fe/kg DM. Fry (2011) found approximately a 2-fold increase in

the mRNA expression of the hepatic Cu efflux pump (ATP7b) in high Fe fed calves which is responsible for biliary Cu excretion and incorporation of Cu into Cp (Kim *et al.*, 2008). In the current study there was an effect of time on Cp and Cp:pl-Cu and effect of time x Fe on Cp which was decreased by time in all treatments but was higher in lambs fed Fe supplemental diet, the reason behind this is not clear.

The decrease observed in the activity of SOD of lambs given Fe supplemented diet are in agreement with the result reported by Humphries *et al.* (1983), when erythrocyte SOD activity and plasma Cu concentrations was significantly decreased in calves given Fe supplemental diet (800 mg Fe/kg) after 16 weeks, and further decreases were observed on week 32. However, these results disagree with those reported by Williams (2004) in sheep given 500 mg Fe/ kg for 12 weeks or with the results reported by Bremner *et al*. (1987) in pre-ruminant calves given different levels of dietary supplemented Fe (730, 1460 and 2190 mg Fe/kg DM, as $FeCl_3$) for 12 weeks, but a significant reduction were observed in weaned calves at week 30. The results also disagree with those reported by Grace *et al*. (1997), who reported a significant reduction in the SOD activity of lambs given high S supplemented diets. The significant effect of time or time x Fe on SOD activity was observed but was not very severe as the time of the study was not long enough. Red blood cell have a long lifespan which exceeds 120 days in sheep (Kurata *et al.,* 1993); therefore the period of experiment may have not be enough to shows the effect on RBCs count. This result observed in the current study is also in agreement with the results observed in Chapter 3 and with the result reported by Williams (2004) in sheep fed diet supplemented with 500 mg Fe/kg DM. The high Hb and Hct results obtained in the group fed diet supplemented with Fe disagree with the results reported by Williams (2004). The results are within the normal ranges reported for sheep (Wolfensohn and Lloyd, 1998). There was a significant effect of time on RBC, WBC, and Hb of lambs which is not of biological importance as they were within normal ranges reported for sheep.

4.5. Conclusion

In conclusion, the results obtained from the current experiment showed that dietary S with or without Fe has no effects on performance or health of growing lambs. High dietary Fe, but not S, reduced liver and plasma Cu concentration significantly. Iron supplements increased liver Fe and plasma Mo concentration but had no effects on liver Mn, Mo, and Zn concentration. Dietary Fe had no effect on biliary Cu concentration but increased biliary Mo concentration and there was Fe x S interaction on biliary Mo concentration of lambs. There was no significant Fe x S interaction on liver mineral concentration of growing lambs; however there was a trend (P =0.06) for Fe x S interaction to reduce liver Cu concentration. Iron supplemental diet increase Cp and Cp:Pl-Cu ratio of lambs and S supplemental diet reduced Cp and Cp:Pl-Cu ratio. Activity of SOD also decreased by feeding high Fe diet but dietary S level had no effect on SOD activity of growing lambs.

CHAPTER 5

The effect of breed and iron supplementation on the liver and blood copper status of growing lambs

5.1. Introduction

Copper deficiency is a widespread problem in sheep and cattle (Underwood, 1977) and can be manifest when dietary Cu supply is less than requirements (NRC, 2007). Copper deficiency is common when excessive amounts of Mo, S, or Fe, are present in diets (NRC, 2005). Breed differences can also affect Cu metabolism and may contribute to this issue (Fry *et al*., 2013). The existence of genetic differences between different breeds in Cu metabolism was studied by many researchers in sheep (Wiener *et al*., 1969; Wiener and Field, 1971a; 1971b; Wiener and Field, 1974; Wiener and Woolliams, 1983; Wiener *et al*., 1987) and cattle (Ward *et al*., 1995; Mullis *et al*., 2003; Fry *et al*., 2013). Liver Cu storage of various breeds of sheep were studied previously in Britain and significant variation was observed (Wiener and Field, 1969; Herbert *et al.*, 1978; Woolliams *et al*., 1982). The efficiency of Cu absorption from the digestive tract has been studied by Wiener *et al*. (1978) in different breeds of sheep, and significant differences in the ability of Cu absorption were recorded. Welsh Mountain have a higher ability to absorb and retain Cu than Scottish Blackface (Wiener and Field, 1969; Herbert *et al.*, 1978; Woolliams *et al*., 1983). Texel sheep absorb and retain higher dietary Cu than Finnish Landrace, Scottish Blackface, and other European and British breeds (Woolliams *et al*., 1982; van der Berg *et al*., 1983). Wiener *et al.* (1978) found that North Ronaldsay sheep, from the Orkney Islands, has such a high ability to absorb dietary Cu that the breed will suffer from Cu toxicity on normal sheep diet. Texel sheep could pose a risk of Cu toxicity due to their higher efficient absorption of Cu (Howell, 1996), while Scottish Blackface are less efficient to absorb Cu that could develop Cu deficiency (Wiener *et al.*, 1978; Suttle, 1988).

Determination of the Cu requirements of sheep according to literature has been changing over years and set at 5 mg/kg DM (NRC, 1975) then increased to about 28 mg/kg DM (Suttle, 2010) depending on the physiological status of the sheep. Although significant variation have been found between different breeds of sheep regarding Cu absorption, no consideration for breeds has been made to determine Cu requirements of sheep. There is lack of information about the Cu metabolism between different breeds of sheep in the literature especially Swaledale sheep, a UK hill sheep breed. Therefore, two experiments were conducted to investigate the effects of breed and Fe on Cu metabolism and

performance of Scottish Blackfaces, Swaledale and Texel sheep. Data obtained in chapters 3 and 4 were used determine the level of dietary iron required to reduce copper retention by 50%.

5.2. Materials and methods

All the procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and approved by Harper Adams University Research Ethics Committee prior to the start of the trial.

Two studies were conducted with store lambs to investigate the effects of breed and dietary Fe on liver Cu metabolism and performance characteristics of sheep.

Experiment 1:

In this study, 44 wethers (22 Texel crossbred and 22 Scottish Blackface; initial BW 25.6 ± 1.99 kg), were used in a 2 x 2 factorial design experiment for 6 weeks. To obtain initial liver mineral levels, 8 lambs (4 per breed) were chosen after they were blocked by weight and breed then slaughtered by stunning and exsanguination (first slaughter group) (Table 5.1). Thirty-six remaining lambs were blocked by LW and breed and randomly assigned to one of two dietary treatments (9 lambs per treatment). The lambs were housed in individual pens and bedded on wood shaving in a good ventilated barn throughout the study.

Table 5.1. Liver mineral concentration of initial slaughter lambs (mg/kg) (n=8 ±SD)

Mineral	SB	T	*P-value*
Cu	301 ±126.3	309 ±103.5	0.93
Fe	494 ±149.7	659 ±113.7	0.13
Mn	23.1 ±6.90	26.6 ±3.49	0.40
Mo	2.1 ±0.71	2 ±0.5	0.81
Zn	101.7 ±11.24	98.6 ±9.62	0.69

The formulated basal diet was iso-nitrogenic and iso-energetic that was expected to supply adequate Cu required for growing sheep (NRC, 2007) (Table 5.2). The lambs were purchased through a dealer who stated that the lambs originated from the same farm and were fed a similar diet prior to study for about 2 weeks. The lambs were fed one of the following dietary treatments:

Control: No Fe supplements.

Fe+: Basal diet supplemented with 800 mg Fe/kg DM.

Lambs were assigned to one of four dietary treatments as shown below:

SB - Scottish Blackface lambs given no Fe supplemental diet

SB + Scottish Blackface given Fe supplemented diet

T - Texel crossbred lambs given no Fe supplemental diet

T + Texel crossbred lambs given Fe supplemented diet

Iron was supplemented in the form of iron sulphate $FeSO_4.7H_2O$ (Fisher Scientific., Leicester, UK). The S coming from $FeSO_4$ supplementation was accounted for before mixing the diets. To balance the S contents of both diets, S was supplemented as a reagent grade ammonium sulphate $(NH_4)_2SO_4$ (Alfa Aesar., Ward Hill, USA). All diets were balanced for N using feed grade urea (Trouw Nutrition, Northwich, Cheshire, UK).

Table 5.2. Feed ingredients and chemical composition of the basal diet (study 1)

Ingredient	Basal diet (g/kg)	Fe supplemented (g/kg)
Nutritionally improved straw (NIS)	400	400
Barley	202	202
Sugar beet pulp	150	150
Soya bean meal	150	150
Molasses	50	50
Megalac	20	20
Urea	3	3
Mins/vits premix[1]	25	25
Chemical compotation (g/kg DM)		
DM (g/kg fresh)	865.7	865.7
CP	178.4	175.7
EE	29.2	30.9
NDF	455.1	366.4
Ash	92.4	101.8
ME (MJ/kg DM)[2]	10.2	10.2
MP (g/kg DM)[2]	86.2	86.2
Mineral concentration (mg/kg DM)		
Cu	8.2	8.4
Fe	258	1151
Mo	1.7	1.9
Mn	58.1	67.6
S (g/kg)	4.8	5.1
Zn	66.8	88.8

[1]Mineral premix (25 kg/ ton) (RUMENCO LTD., Burton upon Trent, UK) containing 320,000 IU/kg Vit A, 100,000 IU/kg Vit D3, 2,000 IU/kg Vit E, 18.5% calcium, 2.0% phosphorous, 1.0% magnesium, 12.0% sodium, 25% chloride, 20 mg/kg selenium, 90 mg/kg cobalt, 150 mg/kg iodine, 3000 mg/kg manganese, and 3000 mg/kg zinc.
[2] predicted using equation provided by AFRC (1993)

5.2.1. Experimental routine

Feed was rationed to control lamb growth at rate of 200 g/d (AFRC, 1993). A metric scale was used to weigh the daily feed allowance that was offered twice a day, at 08:00 and 16:00 h, into individual clean plastic buckets, to avoid minerals contamination. Water was available *ad libitum*. Feed samples were collected weekly (~1kg) and stored at -20°C for the subsequent analyses. Feed refusals were recorded twice weekly to calculate the total DM intake.

5.2.2. Live weight determination

Live weights were recorded once a week, on a Monday, at 9:00 h using a weigh crate (Shearwell Data Ltd., Somerset, UK). Metric standard weights were used to calibrate the scale prior weighing for precision and accuracy (F.L. Thornton and Co. Ltd., Wolverhampton, UK). The weekly LW were used to calculate daily feed offered in the following week to achieve a DLWG of 200 g/d (AFRC, 1993).

5.2.3. Blood sampling and analysis

Jugular blood samples were taken once fortnightly, on Thursday, at 11:30 h for plasma and serum as described in Section 2.2. Blood collected in vacutainer K_2EDTA coated tubes designed for trace mineral analysis (Becton Dickinson vacutainer systems, Plymouth, UK) was used for mineral analysis. Blood collected in vacutainers designed for serum collection (silica coated) were stored overnight at 4°C before being centrifuged at 1000g for 10 minutes. Serum was used to determining Cp activity based on the method of Henry *et al*. (1974), using Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in Section 2.5.

5.2.4. Tissue collection and analytical procedures

By the end of week 6, all lambs were slaughtered in a commercial abattoir by electrical stunning and exsanguination similar to the lamb slaughtered on day 0. Whole livers were collected, weighed and samples (~35 g) were taken and stored at -20°C for subsequent analyses. The weekly concentrate samples were defrosted, bulked, and mixed then subsamples were taken and oven dried at 105°C overnight (Binder, Cole-Palmers, UK) (see Section 2.1.1). Dry feed samples were milled using a Delonghi KG79 grinder (Freemans PLC, Sheffield, UK) and analysed in duplicates for CP, EE, ash, and NDF in

according to the procedure provided by Association of Official Analytical Chemist (AOAC, 2016) as described in Section 2.1.3 to 2.1.5 respectively.

Mineral concentration of diets was determined after digestion of dried samples with concentrated HNO_3 (70%; Fisher Scientific., UK) and HCl (37%; Fisher Scientific., UK), using DigiPREP digestion system (QMX Laboratories Ltd., Essex, UK) and analysed by ICP-MS after diluted in 2% HNO_3, 1% methanol, and 0.1% Triton X-100 (Sigma-Aldrich, Dorset, UK) as described by Cope *et al*. (2009) (see Section 2.6). Dry liver samples were analysed following digestion overnight at 60°C in concentrated HNO_3. Plasma mineral concentration was determined after sample dilution (1:20) in 0.5% HNO_3 (Cope *et al*., 2009) (see Section 2.8). Liver and hay reference samples were used to monitor consistency and reliability of specific minerals concentration (European Commission, Rerieseweg, Belgium).

Experiment 2:

In experiment 2, 56 store wethers (28 Texel cross and 28 Swaledale, with an initial BW of 28.2 ± 2.9 kg) were used in a 2 x 2 factorial design experiment. Prior to the commencement of the experiment, 8 lambs per breed were randomly selected and slaughtered, using the same procedure of experiment 1, to assess the initial liver mineral contents of lambs (first slaughter group) (Table 5.3). The 40 remaining lambs were blocked by LW and breed, and then randomly assigned to one of two dietary treatments, 10 lambs per treatment, for 10 weeks. All lambs were housed individually, in metal pens, and bedded on wood shaving in a good ventilated barn throughout the study.

Table 5.3. Liver mineral concentration of the first slaughter lamb group (mg/kg) (n=16 ±SD)

Mineral	Swaledale	Texel	*P-value*
Cu	265 ± 90.7	159 ± 91.2	0.04
Fe	415 ± 91.6	369 ± 123.2	0.41
Mn	49.9 ± 24.3	34.7 ± 8.9	0.12
Mo	4.3 ± 0.5	3.4 ± 0.5	<0.01
Zn	215 ± 55.1	233 ± 63.8	0.55

5.2.5. Diet formulation

The raw feed ingredients used in the current experiment were similar to those used in experiment one except grass pallet nuts were used instead of NIS (Table 5.4). Supplemental feed grade urea (Trouw Nutrition, Northwich, Cheshire, UK) was used to balance N levels in all diets based on N proportion from $(NH_4)_2SO_4$ inclusion to raise the

rumen degradable nitrogen. The diets were mixed on farm using the similar procedure used in experiment 1. The basal diet was formulated to supply adequate level of Cu (NRC, 2007) required for a lamb weighing 30 kg to grow at a rate of 200 g/d (AFRC, 1993). The lambs were fed one of the following diets:

Control: Basal diet.

Fe +: Basal diet supplemented with 368.7 mg Fe/kg DM to obtain a total 800 mg Fe/kg DM.

Lamb groups have fed one of the following dietary treatments:

SW - Swaledale lambs given control diet

SW + Swaledale given Fe supplemented diet

T - Texel lambs given control diet diet

T + Texel lambs given Fe supplemented diet

Supplemental feed grade urea (Trouw Nutrition, Northwich, Cheshire, UK) was used to balance N levels in all diets based on N proportion from $(NH_4)_2SO_4$ inclusion to raise rumen degradable nitrogen.

Table 5.4. Diet formulation and chemical composition of the basal diet (study 2)

Ingredient	Basal diet (g/kg)	Fe supplemented (g/kg)
Dried grass nuts	500	500
Barley grain	200	200
Sugar beet pulp	85	85
Soybean meal	110	110
Molasses	50	50
Megalac	30	30
Mins/vits premix [1]	25	25
Chemical compotation (g/kg DM)		
DM (g/kg fresh)	934.2	935.8
CP	162.4	174.2
EE	24.0	26.6
NDF	406.8	403.3
Ash	89.1	98.5
ME (MJ/kg DM)[2]	11.14	11.1
MP (g/kg DM)[2]	93.82	93.8
Mineral concentration (mg/kg DM)		
Cu	13.6	12.2
Fe	532.4	968.2
S (g/kg)	2.9	2.8
Mn	78.1	58.89
Mo	2.1	2.2
Zn	83.8	78.8

[1]Mineral premix (25 kg/ ton) (RUMENCO LTD., Burton upon Trent, UK) containing 320,000 IU/kg Vit A, 100,000 IU/kg Vit D3, 2,000 IU/kg Vit E, 18.5% calcium, 2.0% phosphorous, 1.0% magnesium, 12.0% sodium, 25% chloride, 20 mg/kg selenium, 90 mg/kg cobalt, 150 mg/kg iodine, 3000 mg/kg manganese, and 3000 mg/kg zinc.

[2] predicted using equation provided by AFRC (1993).

5.2.6. Experimental routine

Lambs were fed at a restricted level, twice a day, at 08:00 and 16:00 h, using a metric scale to support a predicted LWG of 200 g/day (AFRC, 1993). Daily feed was offered in individual clean plastic buckets. Feed samples were collected weekly and stored at -20°C for subsequent chemical analyses. Water was available *ad libitum*. Feed refusals, if present, were recorded twice a week. The LW of lambs was recorded weekly using a weight crate (Shearwell Data Ltd., Somerset, UK). DLWG was calculated by regression analysis based on the weekly LW. The weekly live weights of lambs were used to calculate the daily feed offered in the following week to achieve the targeted DLWG. of 200 g/d (AFRC, 1993).

5.2.7. Blood sampling and analysis

Jugular blood was taken weekly, on a Thursday, at 11:30 h for plasma, serum, and fortnightly for the whole blood. Blood was collected in vacutainer coated with K_2EDTA designed for trace mineral analysis (Becton Dickinson Vacutainer systems, Plymouth, UK) (see Section 2.2). Whole blood samples were collected in 4.0 ml vacutainer, coated with K_2EDTA (Becton Dickinson Vacutainer systems, Plymouth, UK) and run on Vet Haematology Analyser device (Woodley Equipment Co Ltd., Bolton, UK) as described in Section 2.3. Subsamples of whole blood were also taken to determining SOD activity using a Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) as described in Section 2.4. Vacutainers designed for serum collection (silica coated) were stored overnight at 4°C before being centrifuged as described in Section 5.2.3. Serum was used for determining Cp activity based on the method of Henry *et al*. (1974), using Cobas Mira Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK), as described in Section 2.5.

5.2.8. Tissue collection and analytical procedures

By the end of week 10, all lambs were slaughtered in a commercial abattoir using a similar procedure used for the group of lambs slaughtered at day 0. Liver weights were recorded and samples (~ 35 g) were taken and stored at -20°C for further analysis. Weekly concentrate samples were bulked and oven dried as described in Section 2.1.1. Dry feed samples were milled using a Delonghi KG79 grinder (Freemans PLC, Sheffield, UK) and analysed in duplicate for CP, EE, ash, and NDF according to AOAC (2007) as described in Sections 2.1.3, 2.1.4, 2.1.2, and 2.1.5, respectively. Concentrations of mineral in feed was determined as described in Section 5.2.4.

5.2.9. Data analysis

Weekly live weights, blood components, plasma minerals, Cp, Cp:Pl-Cu ratio and SOD activity were analysed by repeated measures analysis of variance with week 0 as a covariate for plasma mineral using GenStat 17th edition (VSN Int. Ltd., Hempstead. UK). All data were analysed by a two way ANOVA as a 2 x 2 factorial design experiment with breed of sheep and dietary treatments being the main factors. Week 0 served as a covariate for plasma mineral concentrations analysis. The significance difference between means was determined using the protected least significant difference (LSD) (Snedecor and Cochran, 1989).

5.3. Results

Experiment 1:

Collection of livers was problematic in the abattoir setting so this has been excluded from the statistical analysis due to unreliable sample identification post slaughter. The liver Cu, Mn, Mo, and Zn content of lambs slaughtered on day 0 were not significantly different between lambs of both breeds (Table 5.1). Texel lambs of the first slaughter group had higher liver Fe concentration compared with Scottish Blackface lambs; however, there was no significant difference between breeds (659.0 vs. 301.0 mg/kg DM).

5.3.1. Feed intake and performance characteristics

Repeated measure analysis of variance showed a significant effect of time on the weekly LW of lambs (P<0.001), but there was no effect of time x Fe, time x breed or time x Fe x breed interaction on weekly LW of lambs. There was no significant diet x breed interaction on lamb LW (Figure 5.1), DLWG, DMI, or FCR of lambs (Table 5.5). Supplemental Fe or breed of sheep also had no effects (P>0.05) on the DLWG (Figure 5.2), total gain, final weight, DMI, or FCR of lambs.

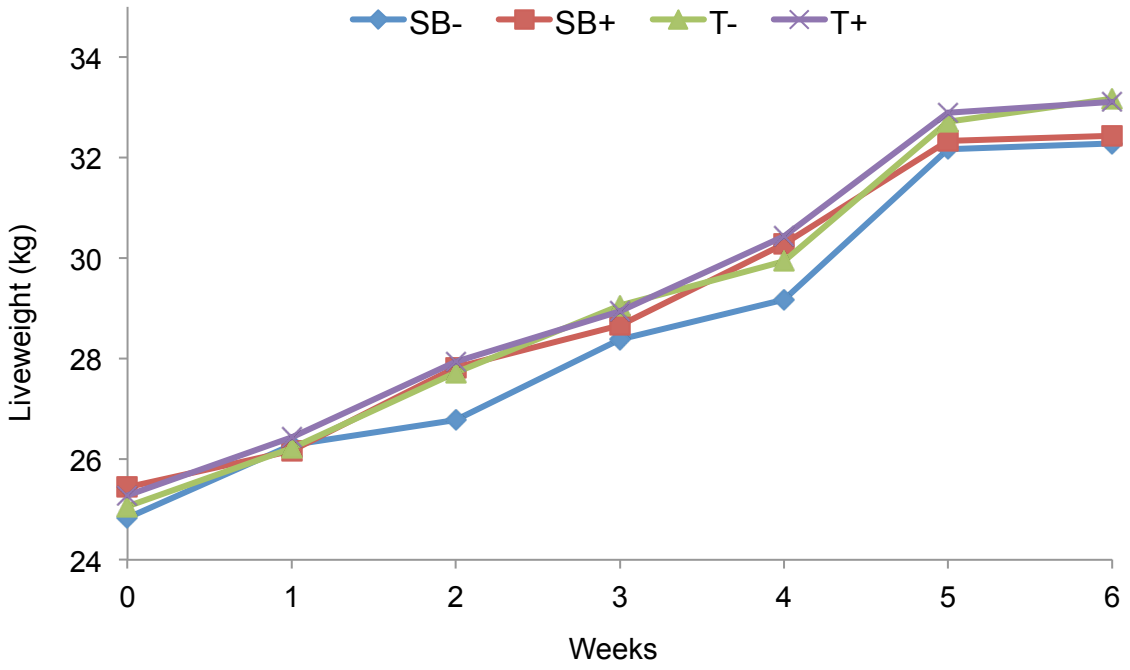

Figure 5.1. Effect of dietary iron and breed on live weight of growing lambs (kg)

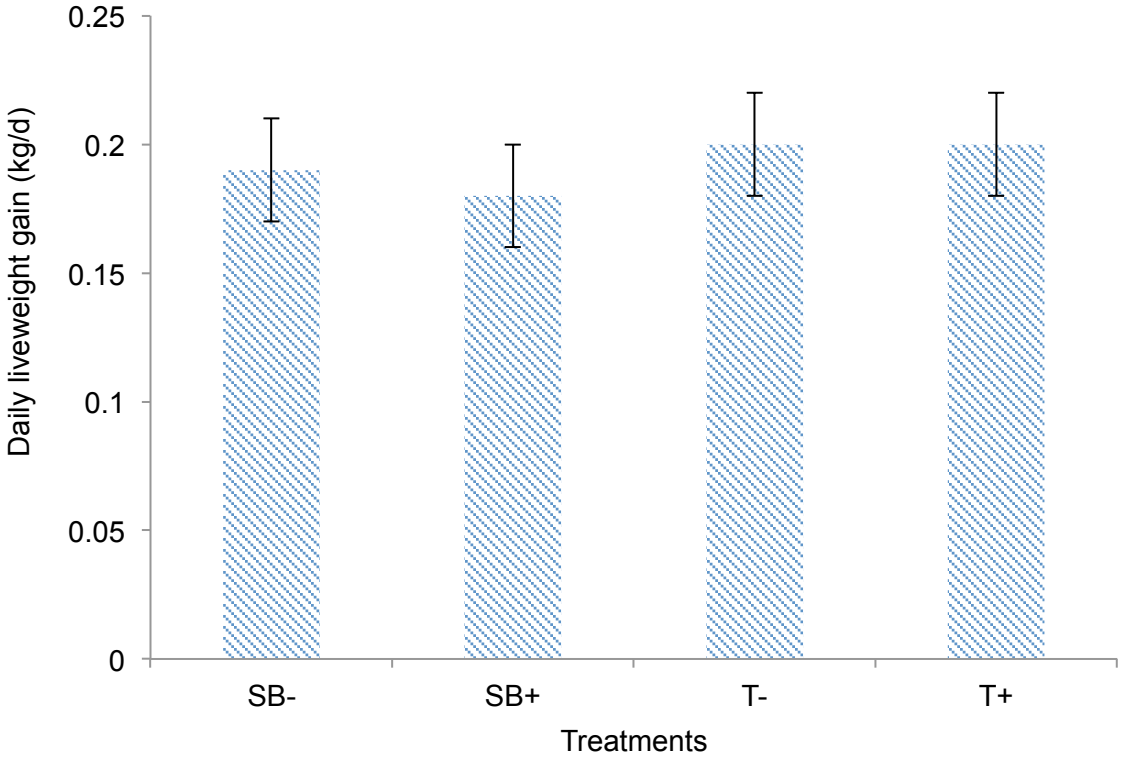

Figure 5.2. Effect of dietary iron and breed on average daily live weight gain characteristic of growing lambs (kg/d). Error bars are s.e.d.

Table 5.5. Effect of dietary Fe and breed of sheep on performance characteristics of growing lambs (kg)

Item	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
Initial wt.	24.8	25.4	25.1	25.3	0.56	0.30	0.94	0.63
Final wt.	32.3	32.4	33.2	33.1	0.87	0.93	0.22	0.86
Total gain	7.4	7.0	8.1	7.8	0.88	0.57	0.24	0.90
DLWG (kg/d)	0.19	0.18	0.20	0.20	0.02	0.76	0.18	0.99
Daily DMI	0.83	0.83	0.86	0.86	0.02	0.72	0.11	0.87
FCR	5.54	5.93	5.26	5.75	0.75	0.42	0.66	0.93

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

5.3.2. Plasma minerals

5.3.2.1. Plasma Copper concentration

Repeated measures analysis of variance showed a significant effect of time on plasma Cu concentration (P<0.001) that was declined with time. There was also a significant time x Fe interaction on plasma Cu concentration (P<0.05), and the lambs fed Fe supplemental diet had lower plasma Cu with time compared to the lambs fed no Fe supplements. There was also a significant time x Fe interaction on plasma Cu concentration (P<0.05). There was no significant time x breed or time x Fe x breed interaction on plasma Cu concentration. Due to differences in the plasma Cu concentration between the two breeds, week 0 was used as a covariate for all plasma mineral ANOVAs. There was no significant Fe x breed interaction on plasma Cu concentration at any weekly time point throughout the study (Table 5.6). The lambs that received supplemental Fe diet had a lower plasma Cu concentration at weeks 2 to 6 compared with the lambs received no Fe. Scottish Blackface lambs had a higher (P<0.05) plasma Cu concentration compared with Texel lambs at week 2, and a trend at week 4 (P =0.07) and 6 (P =0.05).

Table 5.6. Effect of iron supplementation and breed on plasma copper concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	17.11	17.30	14.93	15.49	1.105	-	-	-
2	17.11	15.37	15.57	13.89	0.958	0.01	0.045	0.96
4	14.59	12.32	12.86	11.65	0.840	0.004	0.07	0.36
6	16.25	13.50	14.07	13.07	0.838	0.002	0.05	0.13

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Treatment	0.03
Time x Breed	0.47
Time x Treatment x Breed	0.46

5.3.2.2. Plasma iron concentration

Repeated measures analysis of variance showed a significant effect of time on plasma Fe concentration (P<0.001) that was unclear. There was no significant time x Fe, time x breed or time x Fe x breed interaction on plasma Fe concentration. There was no effect (P>0.05) of Fe x breed interaction on plasma Fe concentration at any weekly time points throughout the study (Table 5.7). Plasma Fe concentration was significantly higher in the lambs given Fe supplements at week 2 (P =0.009), 4 (P =0.01), and 6 (P =0.02) compared with those given no Fe. Texel lambs had lower plasma Fe concentrations (P<0.05) compared with Scottish Blackface lambs at week 4 but not in the following week.

Table 5.7. Effect of iron supplementation and breed on plasma iron concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	38.1	33.2	44.5	42.5	9.27	-	-	-
2	26.5	36.7	28.1	34.0	4.05	0.009	0.86	0.45
4	36.1	41.5	29.1	37.6	3.52	0.01	0.04	0.53
6	38.6	48.6	39.8	47.7	5.00	0.02	0.96	0.77

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.001
Time x Treatment	0.11
Time x Breed	0.18
Time x Treatment x Breed	0.71

5.3.2.3. Plasma manganese concentration

Repeated measures analysis of variance showed no significant effect of time, time x Fe, time x breed or time x Fe x breed interaction on plasma Mn concentration. There was also no significant effect of Fe or Fe x breed interaction on plasma Mn concentration at any weekly time point throughout the study (Table 5.8). Scottish Blackface lambs had a higher plasma Mn concentration (P<0.05) compared with Texel lambs at week 2, but not in the following weeks.

Table 5.8. Effect of iron and breed on plasma manganese concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	0.67	1.03	0.93	0.81	0.162	-	-	-
2	1.17	1.41	1.00	0.96	0.193	0.47	0.03	0.33
4	1.17	1.89	1.31	0.97	0.744	0.76	0.44	0.34
6	0.86	1.15	0.84	0.92	0.186	0.17	0.38	0.43

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.11
Time x Treatment	0.79
Time x Breed	0.58
Time x Treatment x Breed	0.47

5.3.2.4. Plasma molybdenum concentration

Repeated measures analysis of variance showed no significant effect (P>0.05) of time, time x Fe, or time x Fe x breed interaction on plasma Mo concentration. There was a significant time x breed interaction on plasma Mo concentration (P<0.05) with a reduction in plasma levels over the duration of the study observed in the Texel lambs. There was no significant Fe x breed interaction on plasma Mo concentration at any

weekly time points throughout the study (Table 5.9). There was a trend (*P* =0.06) for Fe x breed interaction on plasma Mo concentration at week 6, where plasma Mo concentration was higher in Texel lambs given Fe supplements compared with Scottish Blackface lambs. Plasma Mo concentration was higher (*P*<0.001) in the lambs given supplemental Fe diet compared with the lambs given no Fe supplemental diet from week 2 to 6. Texel crossbred lambs had a higher plasma Mo concentration than Scottish Blackface lambs at week 2 (*P* =0.003) and 6 (*P* =0.001).

Table 5.9. Effect of iron and breed on plasma molybdenum concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	0.11	0.10	0.26	0.40	0.111	-	-	-
2	0.09	0.16	0.15	0.25	0.026	<0.001	0.003	0.10
4	0.11	0.20	0.13	0.25	0.029	<0.001	0.10	0.55
6	0.10	0.18	0.14	0.28	0.025	<0.001	0.001	0.06

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.21
Time x Treatment	0.65
Time x Breed	0.04
Time x Treatment x Breed	0.57

5.3.2.5. Plasma zinc concentration

Repeated measures analysis of variance showed a significant effect of time on plasma Zn concentration (*P* =0.003) which was unclear. There was no significant time x Fe, time x breed, or time x Fe x breed interaction on plasma Zn concentration (Table 5.10). There was also no significant Fe x breed interaction or main effect of Fe and breed on plasma Zn concentration at any weekly time points throughout the experimental period.

Table 5.10. Effect of iron and breed on plasma zinc concentration in growing lambs (µmol/l)

Weeks	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	10.73	13.01	13.49	12.08	0.479	-	-	-
2	15.09	13.97	16.12	15.22	1.114	0.18	0.14	0.99
4	16.40	14.45	16.0	16.25	1.008	0.24	0.25	0.16
6	13.08	13.08	14.30	12.02	1.022	0.31	0.49	0.40

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.003
Time x Treatment	0.32
Time x Breed	0.38
Time x Treatment x Breed	0.32

5.3.3. Ceruloplasmin activity (Cp)

Chapter 7 Repeated measure analysis of variance showed an effect (P<0.001) of time, but no time x Fe or time x Fe x breed on Cp activity. Ceruloplasmin activity was declined with time. There was no significant Fe x breed on Cp activity of lambs (Table 5.11). There was also no main effect (P>0.05) of Fe on Cp activity at any weekly time points throughout the study. At week 2, Scottish Blackface had a higher Cp activity (P<0.05) than Texel lambs but not in the subsequent weeks.

Table 5.11. Effect of iron and breed on ceruloplasmin activity of growing lambs (mg/dl)

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	22.28	20.03	21.01	22.07	2.170	0.70	0.80	0.29
2	29.33	25.91	24.68	23.30	2.062	0.11	0.02	0.49
4	25.12	20.31	19.43	20.09	2.623	0.27	0.12	0.15
6	21.67	18.10	17.44	17.59	2.283	0.30	0.16	0.26

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures:

	P-value
Time	<0.001
Time x Treatment	0.56
Time x Breed	0.12
Time x Treatment x Breed	0.81

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

Repeated measure analysis of variance showed an effect (*P*<0.001) of time, that was unclear, on Cp:Pl-Cu ratio. There was no significant time x Fe, time x breed or time x Fe x breed effect on Cp:Pl-Cu ratio. There was no significant Fe x breed effect on Cp:Pl-Cu ratio at any weekly time points throughout the study (Table 5.12). However, at week 2 there was a trend (*P* =0.08) for Fe x breed on Cp:Pl-Cu ratio that was higher in Texel lambs given the Fe supplemental diet compared to Scottish Blackface given the same diet. There was no significant main effect of supplemental Fe or breed on Cp:Pl-Cu ratio at any weekly time points throughout the study.

Table 5.12. Effect of iron and breed on Ceruloplasmin to plasma copper ratio of growing lambs

Week	Treatments				s.e.d.	*P-value*		
	SB-	SB+	T-	T+		Fe	Breed	FexBreed
0	1.26	1.23	1.40	1.42	0.132	0.96	0.10	0.82
2	1.69	1.57	1.62	1.72	0.082	0.86	0.50	0.08
4	1.69	1.61	1.53	1.73	0.156	0.59	0.88	0.21
6	1.31	1.31	1.25	1.36	0.118	0.51	0.99	0.53

SB-: Scottish Blackface given no Fe supplemental diet, SB+: Scottish Blackface given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures:

	P-value
Time	<0.001
Time x Treatment	0.84
Time x Breed	0.22
Time x Treatment x Breed	0.53

Experiment 2:

5.3.5. Feed intake and performance characteristics

Repeated measure analysis of variance showed a significant effect of time (*P*<0.001) on weekly LW of lambs. There was no breed x time or time x Fe x breed interaction on weekly LW of lambs. There was no significant breed x Fe interaction on weekly LW (Figure 5.3), DLWG, DMI, or FCR at any weekly time point (Table 5.13). Supplementary Fe had no significant effect on the weekly LW or DLWG of lambs. Texel lambs had a higher (*P*<0.05) DLWG than Swaledale lambs (0.155 vs. 0.135 kg/d, s.e.d. =0.008, respectively) and tended (*P* =0.08) to be heavier than Swaledale

by week 10 (Figure 5.4). Breed of lamb had no significant effect on daily DMI or final LW. Texel lambs tended to have higher FCR (*P* =0.08) compared with Swaledale lambs by week 10 (6.87 vs. 8.18 kg, s.e.d. =0.71, respectively).

Table 5.13. Effect of iron and breed on performance characteristics of growing lambs (kg)

Item	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
Initial weight	29.30	29.45	27.15	27.00	0.359	-	-	-
Final weight	39.25	38.17	38.40	38.23	1.488	0.41	0.75	0.54
Total gain	10.20	9.00	11.00	10.95	1.064	0.41	0.08	0.45
DLWG (kg/d)	0.14	0.13	0.15	0.16	0.012	0.98	0.01	0.29
Daily DMI	1.05	1.04	1.05	1.04	0.022	0.41	0.83	0.71
FCR	7.35	9.00	6.99	6.76	1.004	0.33	0.08	0.20

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

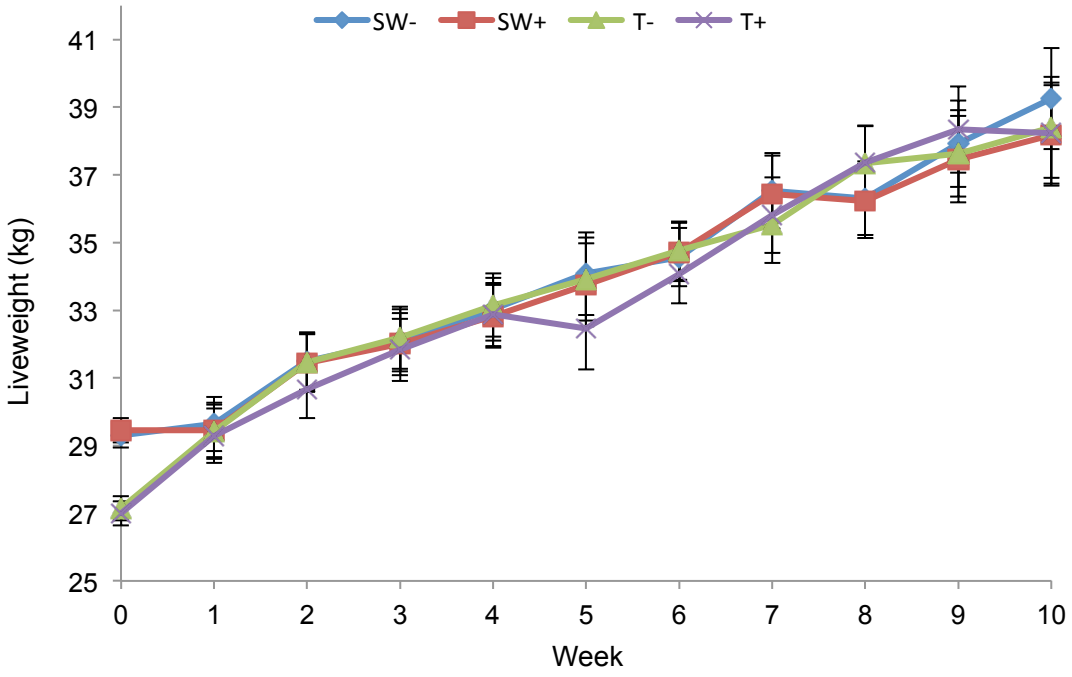

Figure 5.3. Effect of iron and breed on live weight of growing lambs (kg). Error bars are s.e.d.

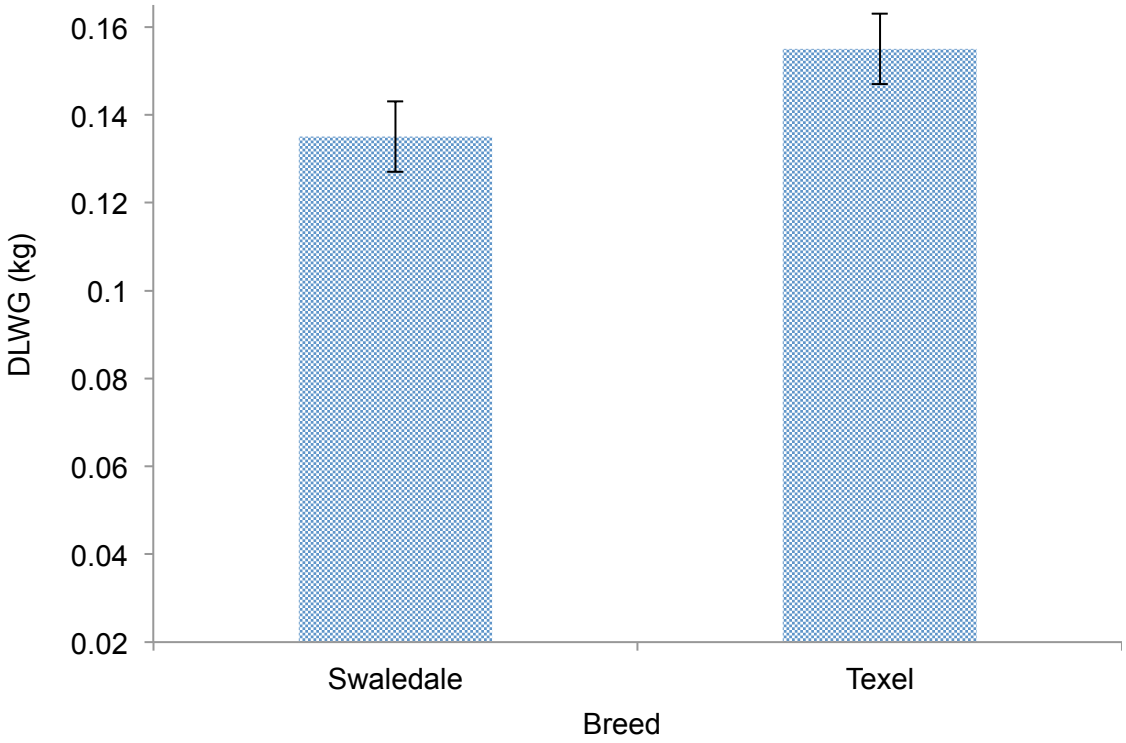

Figure 5.4. Effect of iron and breed on average daily live weight gain characteristic of growing lambs (kg/d). Error bars are s.e.d.

5.3.6. Liver minerals

Swaledale lambs slaughtered at day 0 had a higher (P<0.05) liver Cu concentration (265.0 vs. 159 mg/kg DM, respectively), and higher liver Mo concentration compared with Texel lambs (4.3 vs. 3.4 mg/kg DM, respectively) (Table 5.3). There was no significant difference in the liver Fe, Mn and Zn concentration between both breeds slaughtered at day 0.

There was no significant Fe x breed interaction on liver mineral concentration of lambs. There was also no significant effect of supplemental Fe on hepatic Cu, Fe, or Zn concentration by the end of the study (Table 5.14). The lambs fed Fe supplements had a lower Mn concentration (P =0.08) compared with the lambs fed no Fe (38.3 vs. 49.7 mg/kg DM, s.e.d. =6.22, respectively). The lambs given Fe supplements had significantly lower liver Mo concentration (P<0.001) than lambs given no Fe (3.9 vs. 4.3 mg/kg DM, s.e.d. =0.150, respectively). Texel lambs had significantly higher liver Cu concentration compared with Swaledale lambs (291.0 vs. 187.0 mg/kg DM, s.e.d. =42.0, respectively), but Swaledale lambs had a higher (P<0.05) hepatic Mo concentration compared with Texel lambs (4.43 vs. 3.77, s.e.d. =0.150, respectively). There was no significant effect of breed on liver Fe, Mn or Zn concentration.

Table 5.14. Effect of iron and breed on liver mineral concentration in growing lambs (mg/kg DM)

ID	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
Cu	182.0	191.0	288.0	294.0	59.40	0.86	0.02	0.97
Fe	481.0	557.0	453.0	470.0	84.30	0.45	0.35	0.62
Mn	52.9	32.9	46.6	43.8	8.80	0.08	0.72	0.18
Mo	4.7	4.1	3.8	3.7	0.21	0.02	<0.01	0.15
Zn	151.3	142.2	154.2	160	14.24	0.87	0.32	0.47

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

There was no significant Fe x breed interaction on the total liver mineral content of the lambs at week 10 (Table 5.15). There was also no significant effect of Fe on the total hepatic mineral content. Texel lambs had a significantly higher total liver Cu content compared with Swaledale lambs (44.50 vs. 29.40 mg/liver, s.e.d. =6.87, respectively). However, Swaledale lambs had a significantly higher total liver Mo concentration compared with Texel lambs at (0.70 vs. 0.55 mg/liver, s.e.d. =0.027, respectively). There were no significant differences in the total liver Fe, Mn or Zn content between breeds.

Table 5.15. Effect of iron and breed on the total liver mineral storage in growing lambs (mg/liver)

Mineral	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
Cu	27.8	31.0	43.5	45.5	9.72	0.71	0.04	0.94
Fe	74.7	88.7	64.6	72.3	13.11	0.25	0.17	0.74
Mn	8.24	5.33	6.76	6.52	1.341	0.11	0.88	0.17
Mo	0.74	0.66	0.55	0.56	0.038	0.22	<0.001	0.10
Zn	23.68	22.64	22.46	24.40	2.356	0.79	0.88	0.38

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

5.3.7. Liver mineral balance

There was no significant Fe x breed interaction on the liver minerals balance of lambs (Table 5.16). There was also no significant effect of Fe supplements on liver Cu, Fe, or Zn balance, but lambs supplemented with Fe retained significantly lower Mo per day compared with the lambs fed no Fe supplemental diet (0.0003 vs 0.006 µg/d, s.e.d. =0.0022, respectively). There was a trend for lambs given Fe supplement to retain lower amounts of Mn per day (*P* =0.08) compared with lambs given no Fe

supplemental diet (-0.057 vs. 0.106 µg/d, respectively). Texel lambs retained significantly higher Cu per day compared with Swaledale lambs (1.89 vs. -1.12 µg/d, s.e.d. =0.601, respectively). Texel lambs retained significantly more liver Mn than Swaledale lambs (0.15 vs -0.10 µg/d, s.e.d. =0.089, respectively). There was no significant difference in liver Fe, Mo or Zn balance between Swaledale and Texel lambs.

Table 5.16. Effect of iron and breed on liver mineral balance of growing lambs (µg/d)

Mineral	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
Cu	-1.18	-1.05	1.85	1.93	0.849	0.86	<0.001	0.97
Fe	0.94	2.02	1.21	1.44	1.204	0.45	0.85	0.62
Mn	0.043	-0.243	0.17	0.13	0.126	0.08	0.01	0.18
Mo	0.006	-0.003	0.006	0.004	0.003	0.02	0.13	0.15
Zn	-0.909	-1.04	-1.126	-1.043	0.203	0.87	0.45	0.47

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

5.3.8. Plasma minerals

5.3.8.1. Plasma copper

Repeated measures analysis of variance showed a significant effect of time (P<0.001) on plasma Cu concentration that was lower by the end of the study. There was no significant time x Fe, time x breed, or time x Fe x breed interactions on plasma Cu concentration. Due to differences in plasma Cu concentration between the two breeds, week 0 was used as a covariate for all plasma mineral within time ANOVAs. There was no significant Fe x breed interaction on plasma Cu concentration at any weekly time points through the study (Table 5.17). The lambs fed supplemental Fe diet had significantly lower plasma Cu concentration from week 1 to week 5 compared with the lambs fed no Fe added diet. However, there was no significant effect of supplemental Fe on plasma Cu level from week 6 to 9. There was no difference between Swaledale and Texel lambs on plasma Cu concentration at any weekly time points throughout the study.

Table 5.17. Effect of iron and breed on plasma copper concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	20.86	19.25	22.24	25.13	1.819	-	-	-
1	21.54	18.06	20.60	19.43	1.204	0.01	0.98	0.18
2	20.93	17.44	19.52	18.91	1.281	0.02	0.83	0.12
3	17.50	15.56	18.30	15.79	1.297	0.02	0.57	0.76
4	17.39	15.36	17.33	15.52	0.962	0.01	0.96	0.88
5	16.01	14.55	15.96	15.0	0.808	0.03	0.80	0.67
6	14.82	13.08	14.86	15.73	1.392	0.58	0.29	0.19
7	13.40	12.24	13.33	13.75	1.158	0.59	0.50	0.34
8	16.01	13.09	13.60	14.11	1.749	0.30	0.46	0.17
9	15.74	13.07	13.14	13.30	1.616	0.26	0.25	0.22

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.40
Time x Breed	0.20
Time x Fe x Breed	0.34

5.3.8.2. Plasma iron

Repeated measures analysis of variance showed a significant effect of time on plasma Fe concentration that was unclear. There was no significant time x Fe, time x breed, or time x Fe x breed interaction on plasma Fe concentration.

There was a significant Fe x breed interaction on plasma Fe concentration at week 6; Texel lambs that received the control diet had higher plasma Fe concentration compared with the lambs given any of the other diets, however, there was no difference in plasma Fe concentration between the other treatments (Table 5.18). At week 8 there was a trend for diet x breed interaction (*P* =0.05), with Swaledale lambs fed supplemental Fe and Texel lambs fed no Fe having higher plasma Fe concentration compared with the other groups. There was no effect of breed of lamb on plasma Fe concentration at any sampling time except at week 6 when Texel crossbred lambs tended to have a higher plasma Fe concentration (*P* =0.09) compared with Swaledale lambs (57.1 vs. 50.8 µmol/l, s.e.d. =3.10, respectively).

Table 5.18. Effect of iron and breed on plasma iron concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	32.9	41.4	48.0	43.0	6.10	-	-	-
1	31.2	33.8	35.7	36.7	4.99	0.63	0.33	0.83
2	47.2	40.9	48.4	45.6	8.21	0.43	0.60	0.76
3	43.98	41.12	40.91	41.62	2.918	0.62	0.61	0.40
4	53.7	54.2	54.4	53.7	3.32	0.96	1.00	0.79
5	59.4	52.6	49.3	50.8	7.93	0.66	0.34	0.47
6	49.9^{b}	51.7^{b}	64.1^{a}	50.1^{b}	4.26	0.04	0.09	0.02
7	38.0	40.0	38.0	36.4	3.08	0.91	0.39	0.43
8	36.2	43.3	42.3	37.1	3.80	0.74	0.80	0.05
9	44.1	44.7	54.0	41.4	6.78	0.19	0.60	0.19

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.
[a,b] Means with the same superscript are not significantly different (P<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.57
Time x Breed	0.28
Time x Fe x Breed	0.13

5.3.8.3. Plasma manganese

Repeated measures analysis of variance showed a significant effect of time on plasma Mn concentration that was declined by the end of the study. There was no significant time x breed, time x Fe, or time x Fe x breed interaction on plasma Mn concentration. There was a significant Fe x breed interaction on plasma Mn concentration in which at week 3 Swaledale lambs fed no Fe supplemented diet had a higher (P<0.05) plasma Mn concentration compared with the other treatments (Table 5.19). There was no effect of dietary Fe level on plasma Mn concentration at any weekly time points throughout the study. There was also no effect of breed on plasma Mn concentration at any weekly time points throughout the period of the study.

Table 5.19. Effect of iron and breed on plasma manganese concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	0.92	0.70	0.95	1.08	0.265	0.83	0.28	0.36
1	2.06	1.81	1.87	2.15	0.357	0.96	0.78	0.31
2	1.84	1.48	1.69	1.64	0.420	0.49	0.99	0.61
3	1.21[a]	0.38[b]	0.50[b]	0.64[b]	0.275	0.08	0.22	0.02
4	0.65	0.57	0.64	0.63	0.171	0.70	0.82	0.78
5	0.67	0.52	0.65	0.62	0.135	0.36	0.70	0.56
6	0.61	0.42	0.55	0.62	0.147	0.59	0.52	0.23
7	0.91	1.28	1.16	0.91	0.272	0.77	0.80	0.12
8	1.17	0.89	0.76	0.79	0.222	0.44	0.11	0.33
9	0.68	0.59	0.65	0.53	0.161	0.36	0.68	0.88

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.
[a,b] Means with the same superscript are not significantly different (*P*<0.05).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.14
Time x Breed	0.15
Time x Fe x Breed	0.54

5.3.8.4. Plasma molybdenum

Repeated measures analysis of variance showed a significant effect of time (*P*<0.001) that was declined with time, time x Fe (*P*<0.05) were the lambs fed supplemental Fe had higher plasma Mo than those fed non supplemented with time, and time x breed (*P*<0.05) were Texel lambs had higher plasma Mo concentration in all weeks. There were no significant time x Fe x breed interactions on plasma Mo concentration. There was also no significant Fe x breed interaction on plasma Mo concentration at any time point throughout the study. Dietary supplemental Fe had no significant effect on plasma Mo concentration at any weekly time point throughout the study (Table 5.20). In week 7, plasma Mo concentration tended to be lower (*P* =0.08) in the group given no Fe supplemental diet compared with those fed Fe supplemental (0.166 vs. 0.150 µmol/l, s.e.d. =0.011, respectively). Texel lambs had a higher plasma Mo concentration (*P* =0.09) compared with Swaledale lambs in week 2, and had a higher plasma Mo

concentration in weeks 4, 5, 9, (P<0.01) and in weeks 6, 7, and 8 (P<0.05) compared with Swaledale lambs.

Table 5.20. Effect of iron and breed on plasma molybdenum concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	0.21	0.22	0.35	0.30	0.043	-	-	-
1	0.13	0.16	0.17	0.17	0.020	0.39	0.18	0.23
2	0.14	0.16	0.17	0.19	0.020	0.21	0.09	0.98
3	0.15	0.16	0.16	0.17	0.019	0.78	0.54	0.96
4	0.12	0.13	0.17	0.17	0.013	0.62	<.001	0.92
5	0.14	0.13	0.16	0.18	0.016	0.73	0.004	0.40
6	0.13	0.13	0.17	0.17	0.017	0.72	0.01	0.74
7	0.13	0.14	0.16	0.19	0.018	0.08	0.01	0.53
8	0.23	0.22	0.31	0.26	0.027	0.11	0.02	0.26
9	0.16	0.17	0.19	0.21	0.017	0.14	0.004	0.56

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.01
Time x Breed	0.03
Time x Fe x Breed	0.14

5.3.8.5. Plasma zinc

Repeated measures analysis of variance showed a trend (P =0.06) for the effect of time on plasma Zn concentration. There was no significant time x Fe, time x breed, or time x Fe x breed interaction on plasma Zn concentration. There was no significant Fe x breed interaction on plasma Zn concentration at any weekly time point throughout the study (Table 5.21). The lambs that received supplemental Fe had significantly lower plasma Zn concentration at weeks 3, 4, and 5 compared with those given no Fe supplemental diet. At week 9, the lambs given supplemental Fe diet tended to had lower plasma Zn concentration (P =0.06) compared with lambs given control diet. Breed of lamb had no significant effect on plasma Zn concentration at any weekly time points through the study.

Table 5.21. Effect of iron and breed on plasma zinc concentration in growing lambs (µmol/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	10.47	11.89	12.81	12.54	0.750	-	-	-
1	10.81	10.77	11.95	10.68	0.630	0.10	0.37	0.17
2	11.72	11.45	11.43	11.37	0.537	0.68	0.67	0.78
3	11.99	10.83	11.45	10.88	0.568	0.04	0.64	0.46
4	13.10	12.41	12.89	11.96	0.498	0.03	0.36	0.72
5	12.85	11.43	12.19	11.46	0.524	0.01	0.51	0.35
6	12.09	11.97	11.94	11.0	0.548	0.18	0.15	0.29
7	9.45	9.36	9.62	8.59	0.707	0.25	0.49	0.34
8	12.88	12.59	12.14	11.74	0.665	0.53	0.12	0.90
9	12.51	11.57	11.81	10.53	0.788	0.06	0.14	0.76

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.06
Time x Fe	0.35
Time x Breed	0.27
Time x Fe x Breed	0.38

5.3.9. Ceruloplasmin activity (Cp)

Repeated measure analysis of variance showed a significant effect of time that was reduced with the progress of the study, but not time x Fe, time x breed and time x Fe x breed interaction on Cp activity. At week 1 and 2, there was a trend for Fe x breed interaction on Cp activity of lambs (*P*= 0.07 and *P*= 0.05, respectively); Swaledale lambs given supplemental Fe had a lower Cp activity compared with any other treatments and this became significantly different (*P*<0.05) at week 3. There was no significant Fe x breed interaction on Cp activity from week 4 to week 9 (Table 5.22). There was no significant effect of supplemental Fe diet on Cp activity at any weekly time point throughout the study. At week 4, the lambs fed Fe supplemental diet tended (*P* =0.05) to have lower Cp activity than lambs fed the control diet (19.15 vs. 21.66 mg/dl, s.e.d. =1.196, respectively). There was no significant effect of breed of sheep on Cp activity of lambs at any weekly time point throughout the study.

Table 5.22. Effect of iron and breed on ceruloplasmin activity of growing lambs (mg/dl)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T -	T +		Fe	Breed	FexBreed
0	23.94	20.34	28.99	29.51	2.282	-	-	-
1	26.60	21.77	23.98	24.24	2.051	0.10	0.70	0.07
2	22.27	18.43	20.03	21.81	2.224	0.53	0.98	0.05
3	21.63[a]	17.41[b]	21.08[a]	23.22[a]	2.334	0.63	0.31	0.04
4	22.28	18.28	21.04	20.02	1.882	0.05	0.97	0.23
5	19.76	18.86	22.18	21.24	1.868	0.56	0.13	0.99
6	17.58	17.70	17.85	21.60	2.731	0.22	0.45	0.31
7	17.30	16.99	18.44	19.28	2.178	0.78	0.39	0.68
8	20.00	17.20	13.90	15.40	3.560	0.69	0.13	0.35
9	18.60	17.10	14.80	16.30	3.410	0.94	0.36	0.48

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.
[a,b] Means with the same superscript are not significantly different ($P<0.05$).

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.31
Time x Breed	0.56
Time x Fe x Breed	0.44

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

Repeated measure analysis of variance showed a significant effect of time (P<0.001) on Cp:Pl-Cu ratio that was unclear. There was no significant time x Fe, time x breed or time x Fe x breed interaction on Cp:Pl-Cu ratio.

There was also no significant Fe x breed interaction on Cp:Pl-Cu ratio at any weekly time points throughout the study (Table 5.23). However, at week 7, there was a trend (P= 0.08) for a Fe x breed interaction on the Cp:Pl-Cu ratio; which was lowest in SW lambs. Supplemental Fe had no effect on Cp:Pl-Cu ratio from week 1 to 5 and week 7 to 9, but the lambs given Fe supplemented diets had a significantly higher Cp:Pl-Cu ratio compared with lambs fed control diet (1.35 vs. 1.18, s.e.d. =0.080, respectively) at week 6. There was no effect of breed on Cp:Pl-Cu ratio at any weekly time points throughout the study.

Table 5.23. Effect of iron and breed ceruloplasmin to plasma copper ratio of growing lambs

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T -	T +		Fe	Breed	FexBreed
0	1.142	1.051	1.305	1.212	0.063	-	-	-
1	1.213	1.198	1.162	1.224	0.042	0.47	0.72	0.17
2	1.051	1.060	1.022	1.126	0.054	0.11	0.65	0.18
3	1.256	1.199	1.165	1.354	0.117	0.37	0.73	0.11
4	1.280	1.215	1.188	1.255	0.070	0.93	0.64	0.15
5	1.242	1.332	1.353	1.373	0.073	0.17	0.19	0.46
6	1.165	1.368	1.187	1.337	0.116	0.03	0.95	0.72
7	1.129	1.350	1.408	1.318	0.136	0.35	0.25	0.08
8	1.145	1.184	1.059	1.174	0.132	0.44	0.65	0.65
9	1.100	1.231	1.148	1.228	0.129	0.22	0.83	0.76

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.58
Time x Breed	0.58
Time x Fe x Breed	0.16

5.3.11. Effects on superoxide dismutase activity (SOD)

Repeated measure analysis of variance showed a significant effect of time (*P* =0.002) that was unclear, but not time x breed, time x Fe or time x Fe x breed on SOD activity of lambs. There was no significant effect of Fe x breed interaction on SOD activity at any weekly time point throughout the study (Table 5.24). There was also no effect of Fe supplements on SOD activity at any weekly time point throughout the study. There was also no effect of breed on SOD activity at any weekly time point throughout the study except at week 9, where Swaledale lambs had a higher SOD activity (*P*<0.05) compared with Texel lambs.

Table 5.24. Effect of iron and breed on superoxide dismutase activity of growing lambs (USOD/g Hb)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T -	T +		Fe	Breed	FexBreed
0	2011	2171	1765	2558	217.0	-	-	-
2	2246	2452	2323	2526	234.4	0.26	0.63	0.99
4	2159	2212	1929	2097	159.6	0.38	0.11	0.61
6	2172	2368	2098	2224	207.3	0.27	0.43	0.81
9	2509	2659	2210	2281	241.3	0.45	0.04	0.82

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.002
Time x Fe	0.23
Time x Breed	0.26
Time x Fe x Breed	0.25

5.3.12. Blood components

5.3.12.1. Red (RBCs) and white (WBCs) blood cells

Repeated measures analysis of variance showed no significant effect of time, time x Fe, time x breed, or time x Fe x breed interactions on RBC count. There was no significant diet x breed interaction on RBC count of lambs at any weekly time point throughout the experiment period (Table 5.25). There was also no significant effect of supplemental Fe on RBC count throughout the study until week 9, when the lambs receiving supplemental Fe had a higher (P<0.05) RBC count compared with lambs fed no Fe. Texel lambs had significantly lower RBC count compared with Swaledale lambs at week 4 (P<0.05) and week 9 (P =0.002).

Table 5.25. Effect of iron and breed on red blood cell count in growing lambs (x10^{12}/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
2	11.96	14.88	11.55	11.74	1.607	0.18	0.13	0.24
4	13.45	14.04	12.96	13.08	0.459	0.29	0.04	0.47
6	12.49	13.70	12.10	12.48	0.756	0.15	0.15	0.44
9	13.55	13.89	12.18	13.18	0.439	0.04	0.002	0.30

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.10
Time x Fe	0.65
Time x Breed	0.67
Time x Fe x Breed	0.24

Repeated measures analysis of variance indicated a significant (*P*<0.001) effect of time on WBC count that was unclear. There was no effect of the time x breed, time x Fe, or time x Fe x breed interaction on the WBCs count. There was also no significant effect of Fe or Fe x breed interaction on WBC throughout the study (Table 5.26). Texel lambs had a higher WBC count compared with Swaledale lambs in week 6 (*P*<0.05) and at week 9 (*P* =0.07).

Table 5.26. Effect of iron and breed on white blood cell count in growing lambs (x10^{9}/l)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
2	10.47	10.40	11.37	10.47	1.120	0.55	0.55	0.60
4	8.99	9.33	9.74	10.33	0.795	0.42	0.13	0.82
6	8.35	8.14	9.53	9.95	0.844	0.86	0.02	0.60
9	9.06	9.77	10.06	12.50	1.387	0.12	0.07	0.39

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.45
Time x Breed	0.20
Time x Fe x Breed	0.32

5.3.12.2. Effects on Haematocrit (Hct)

Repeated measures analysis of variance showed a significant effect of time, that was increased with time, and time x breed interaction on Hct percentage (*P*<0.001), were SW lambs had higher Hct than Texel lambs in all weeks. There was no significant effect of time x Fe or time x Fe x breed interactions on Hct. There was no significant Fe x breed interaction on Hct % at any weekly time point throughout the study (Table 5.27). Dietary Fe had no effect on Hct % throughout study (*P*>0.05). Swaledale lambs had a higher Hct % compared with Texel lambs at week 6 and week 9 (*P*<0.05).

Table 5.27. Effect of iron and breed on haematocrit in growing lambs (%)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
2	33.28	33.69	33.17	33.29	1.845	0.84	0.85	0.91
4	38.98	40.66	38.37	38.12	1.780	0.58	0.22	0.45
6	36.93	39.31	35.57	35.74	1.675	0.29	0.004	0.36
9	39.55	40.98	36.75	38.97	1.578	0.11	0.04	0.73

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	<0.001
Time x Fe	0.67
Time x Breed	<0.001
Time x Fe x Breed	0.40

5.3.12.3. Effects on Haemoglobin (Hb)

Repeated measure analysis of variance showed no significant effect of time, time x breed, time x Fe, or time x Fe x breed interaction on Hb concentration. There was no significant Fe x breed interaction or main effect of supplemental Fe on Hb concentration throughout the study (Table 5.28). There was also no effect of breed on Hb concentration throughout the study except at week 4, when Swaledale lambs had a significantly higher Hb concentration compared with Texel lambs (14.0 vs. 13.42 g/dl, s.e.d. =0.284, respectively).

Table 5.28. Effect of iron and breed on haemoglobin in growing lambs (g/dl)

Week	Treatments				s.e.d.	*P-value*		
	SW -	SW +	T-	T+		Fe	Breed	FexBreed
0	12.54	12.82	11.81	11.02	0.578	-	-	-
2	13.27	13.15	12.53	13.14	0.445	0.38	0.22	0.24
4	14.04	13.97	13.19	13.65	0.362	0.35	0.04	0.29
6	14.52	13.47	11.69	13.23	1.669	0.78	0.18	0.26
9	13.19	13.80	12.63	12.54	1.245	0.69	0.39	0.69

SW-: Swaledale given no Fe supplemental diet, SW+: Swaledale given Fe supplemental diet. T-: Texel given no Fe supplemental diet, T+: Texel given Fe supplemental diet.

Repeated measures analysis:

	P-value
Time	0.38
Time x Fe	0.96
Time x Breed	0.47
Time x Fe x Breed	0.14

5.4. Discussion

The level of Fe used in experiment 5.1 was chosen depending on the results obtained from chapter 3 and 4 to decrease liver Cu retention by 50%. The Fe content of the basal diet used in experiment 5.1 was within the normal published range in animal diets (257.46 mg/kg DM). Experiment 5.2, was designed to study the effect of 800 mg Fe/kg DM total Fe, but the chemical analysis of the basal diet showed a higher Fe concentration than the predicated values (532.4 mg/kg DM) from MAFF (1992). The high Fe level of the basal diet used in experiment two was due to the high Fe contents of the grass pellets and sugar beet pulps used (623.74 and 574.12 mg/kg DM, respectively). The mean Fe concentration of grasses has been reported to average 103 mg/kg DM in the UK, (ranges 73–154 mg/kg DM) (Whitehead, 1966). The forage Fe concentration is influenced by changes in the soil conditions, climate and stage of growth (Givens *et al*., 2000). Grass grown on soils derived from serpentine had Fe concentrations ranging from 2127 to 3580 mg/kg DM (Johnston and Proctor, 1977), but values beyond 300 mg/kg DM generally reflect soil contamination of the sample, rather than actual Fe content in the forages (Givens *et al*., 2000). From this information, it can be assumed that the high level of Fe in both grass nuts and sugar beet pulps was due to soil contamination. Very little is known on the Fe bioavailability of forage (Hansen and Spears, 2009). The Fe availability of soil is reported to be low unless it is subject to an acidic condition such as that of silage during fermentation (Hansen and Spears, 2009).

Supplemental Fe in both experiments had no effect on performance of lambs such as DLWG, total gain, daily DM intake, or FCR, similar to the results observed in chapter 3 and 4. The liver mineral concentration of the initial slaughter group of the first experiment showed no significant differences in liver mineral concentration of both breeds, which may have been due to the effects of boluses containing Cu that were fed to the dams of Scottish Blackface, as reported by the supplier. In experiment two, the variation in mineral metabolism between both breeds was more obvious although the supplier mentioned that they had been reared under the same condition.

The initial hepatic Cu concentration of Texel lambs was lower than that of Swaledale lambs that could be due to the pre-experiment dietary treatments, but was within the normal range (159.0 and 265.0 mg/kg DM, respectively) (NRC, 2005), although the Texel breed has been reported to be more efficient at Cu absorption and retention compared with other British breeds (Woolliams *et al*., 1982; Suttle *et al*., 2002). The information available on Cu metabolism of Swaledale sheep is scarce and they are assumed to have a similar metabolism model to other highland breeds. The increase observed in the liver Cu concentration of Texel lambs at the end of the second study was similar to that

reported by other researchers (Woolliams *et al*., 1982; van der Berg *et al*., 1983; Littledike and Young, 1993; Suttle *et al*., 2002) who found a high efficiency of Texel lambs to absorb and retain Cu compared to other breeds. On the other hand, Swaledale lambs have been observed to behave more like hill breeds and retain less hepatic Cu than Texel lambs, although they had a higher initial liver Cu concentration than Texel lambs. In the current study a dietary supplementation of 368.7 mg Fe/kg DM had no effect on the liver Cu storage of lambs. This could have been due to the low level of supplemental Fe on Cu status of sheep although the total Fe level of the basal diet was high (532.4 mg/kg DM). Several researchers found a significant reduction in the liver Cu concentration of sheep and cattle (Phillippo *et al*., 1987a; Prabowo *et al*., 1988; Williams, 2004) fed Fe supplemented diets (500 – 800 mg Fe/kg DM) and also the data reported in Chapter 3 and 4 has confirmed this effect. Phillippo *et al*. (1987a) in cattle and Prabowo *et al*. (1988) and Williams (2004) in sheep have used a higher level of supplemental Fe, but in the current experiment the amount of supplemental Fe was lower and may have not been enough to exert its maximum effects on reducing liver Cu concentration. In addition, significant effect of supplemental Fe were found in lambs used in Chapter 3 and 4 when the diet was supplemented with over 500 mg Fe/kg DM. On the other hand, the current findings are in agreement with the result reported by Rosa *et al.* (1986) in sheep who did not find an effect of dietary Fe supplements (1000 mg Fe/kg DM, as $FeCl_3$) on the liver Cu storage of mature sheep. If the Fe content of the basal diet was not completely available, then the results obtained in the current study are in accordance with those observed in Chapter 3 when 250 mg supplemental Fe per kg DM had no significant effect on liver Cu concentration of lambs. No clinical sign of Cu deficiency was found in either experiment. Clinical sings of Cu deficiency can be observed when the liver Cu concentration drops to <20 mg/kg DM, which is set as the threshold for Cu deficiency estimated by NRC (2007), or to <10 mg/kg DM by Suttle (2010). This result is in agreement with the results observed in Chapters 3 and 4 and also with the results of Humphries *et al.* (1983) who found no clinical signs of Cu deficiency in calves when the diet was supplemented with 800 mg Fe/kg DM after 32 weeks. Similarly, Williams (2004) and Prabowo *et al*. (1988) did not found effects of Fe supplements Fe on clinical signs of Cu deficiency in sheep.

Similar to the results observed in Chapters 3 and 4, supplemental Fe had no effect on liver Zn concentration of the lambs. However, Williams (2004) observed a significant reduction in the hepatic Zn concentration of lambs when fed a diet supplemented with 500 mg Fe/kg DM. Yamaji *et al*. (2001) reported that high dietary Fe concentrations had a limiting effect on Zn absorption. Divalent metal transporter (DMT1) is responsible for Fe uptake in the small intestine (Yamaji *et al.,* 2001), and it also suggested to be involved with the uptake of Zn beside Zip4 (NRC, 2005). Therefore, increases in the dietary Fe concentration may have been competing with Zn absorption from the small intestine.

Standish *et al*. (1971) found a significant reduction in the liver Zn concentration of beef calves given a diet supplemented with 1000 mg Fe/kg DM. Lönnerdal (2000) found that dietary supplementation of Fe together with Zn reduced Zn absorption in simple stomach species. The decrease in the liver Mn concentration observed in the current study disagrees with the results reported in Chapter 3 and 4 or with the results observed by Williams (2004) in sheep. On the other hand, these finding are in accordance with the results observed in cattle fed high levels of dietary Fe (Hansen and Spears, 2009). Hansen and Spears (2009) reported that both Fe and Mn share the same intestinal protein transporter DMT1 and a high dietary Fe concentration may compete with Mn absorption from intestine. The hepatic Fe concentration of the lambs fed Fe supplements increased but not significantly, indicating that the availability of Fe from the supplementary Fe diet was higher compared with those fed the control diet. These results are in accordance with those observed in Chapter 3 when lambs fed a diet supplemented with 250 mg Fe/kg DM, but not with those given 500 or 750 mg Fe/kg DM. Supplemental Fe reduced liver Mo concentration in lambs in the current trial unlike the result reported by Williams (2004) in lambs given diets supplemented with 500 mg Fe/kg DM or with the results observed in chapter 3 when no effect of supplemental Fe on liver Mo concentration was observed. The high liver Mo content of Swaledale lambs remained higher than that of the Texel lambs from day 0 to the end of the study.

The reduction in plasma Cu concentration of the lambs given Fe supplements in both experiments is in agreement with the result observed in Chapter 4 and also with the results reported by other researcher in sheep (Suttle *et al*., 1984; Prabowo *et al.,* 1988; Williams, 2004) and cattle (Standish *et al.,* 1969; Humphries *et al*., 1983; Bremner *et al*., 1987; Phillippo *et al*., 1987a).

In the second but not in the first experiment, it was observed that the initial plasma Cu concentration was high but within the ranges reported for sheep that may have Cu toxicity although the liver Cu concentration of the lambs was normal. Plasma Cu concentrations >21.24 µmol/l have been reported to indicate Cu poisoning in sheep (Osweiler and Carson, 1985). The normal ranges of ovine plasma Cu concentration reported in UK is 9.4 – 19 µmol/l with an average of 15 µmol/l (Rushton, 1981). The regulation of blood Cu concentrations in sheep is not well understood, but it is probably under homeostatic control at the level of the liver Cu stores. When the hepatic Cu stores are sufficient, blood Cu concentrations remain relatively constant (Herdt and Hoff, 2011). Inflammation has been reported to have an up-regulating impact on blood Cu concentrations; this effect is due to Cp concentrations responding as an acute-phase protein, increasing during conditions of a generalised inflammatory response constant (Herdt and Hoff, 2011). No disease was found so this effect is unexplained. Suttle *et al*. (1984) suggested that high

dietary Fe may combine with S within the rumen to form an Fe-S complex, then Cu exchanges with S in the acid environment of abomasum resulting in the formation un-absorbable Fe-Cu complex. This reaction between aforementioned minerals reduces Cu absorption and may lead to a decrease in plasma Cu concentrations. No clinical signs of Cu deficiency was observed in either studies as the plasma Cu concentration of lambs was higher than 9 µmol/l (Suttle, 1986). Plasma Cu concentration decreased by 9.9% in Texel lambs fed Fe supplements compared with Texel given no Fe, but in Scottish Blackface lambs feds diet supplemented with 800 mg/kg DM it was reduced by 16.9% compared with those fed no Fe after 6 weeks. In experiment two, plasma Cu concentration reduced less by feeding 800 mg Fe/kg DM; Swaledale lambs fed the Fe supplemented diet had plasma Cu concentration 7.6% lower than Swaledale lambs given same control diet and Texel lambs had 6.2% lower plasma Cu given the same diet after 10 weeks. The non-significant differences in the plasma Cu of Fe fed lambs in experiment two, from week 6 to week 9 is unclear. Liver Cu can be released into plasma spontaneously or at the times of stress, including shearing, weather extremes or transport (Jones and Van Der Merwe, 2008).

Differences in Cu metabolism between Texel and Scottish Blackface lambs in the first study was clear and showed the effect of Fe antagonist on plasma Cu concentration of either breeds which is in accordance with the results reported by other researchers (Wiener *et al*., 1969; Wiener and Field, 1971a; 1971b; Wiener and Field, 1974; Wiener and Woolliams, 1983; Wiener *et al*., 1987) and cattle (Ward *et al*., 1995; Mullis *et al*., 2003; Fry *et al*., 2013). Scottish Blackface lambs had a higher plasma Cu concentration throughout the study compared with Texel lambs. On the other hand, no effects of breed of sheep on plasma Cu concentration were observed in the second experiment.

Fe supplementation in both studies had no effect on plasma Mn concentration. In Experiment 1, the lambs that given dietary supplemental Fe had a higher plasma Fe concentration than those fed no Fe supplements, which is in agreement with the results observed in Chapter 3 and 4 and with those reported by Phillippo *et al*. (1987a), Humphries *et al*. (1983), and Bremner *et al*. (1987) in cattle given Fe supplemented diets which was also associated with an increases in liver Fe concentration. However, Prabowo *et al*. (1988) observed that plasma Fe in blood of sheep obtained after 84 days was not consistently affected by dietary Fe. The plasma Fe level increased in experiment 1 from week 2 in lambs given supplemental Fe, indicating the highly availability of the Fe form used (Hurrell *et al*., 1989). Plasma Fe concentration in the second study was not significantly altered by either dietary treatment or breed of sheep, which could have been due to the low level of the supplemental Fe in the Fe supplemented diet.

The high plasma Mo concentration of the lambs given supplemental Fe observed in experiment 1 is similar to the result observed in Chapters 3, and 4. The majority of the body losses of Mo take place through the urinary excretion after transportation via the blood (NRC, 2005). In Chapter 3, it was observed that increasing dietary Fe resulted in increased Mo in both plasma and urine. Breed of sheep had no effect on plasma Mo level in experiment 1 but in experiment 2, Texel lambs had a higher plasma Mo concentration than Swaledale lambs although hepatic Mo content of Swaledale lambs was higher than those of Texel lambs before and after feeding the experimental diets. There was no effect of Fe supplementation on plasma Mo level in experiment 2, which again shows the breed difference effect on Mo metabolism. Plasma Zn concentration was not influenced by supplemental Fe in experiment 1, which is in agreement with the result obtained in Chapters 3, 4. However, in experiment 2 the lambs that were given the Fe supplemental diet had a lower plasma Zn concentration than those given no Fe supplements in week 3, 4, and 5.

Similar to the result observed in Chapter 3 and 4, time had an effect on the plasma Cu and Zn concentration in experiment 5.1 and on Cu, Mn and Mo in experiment 5.2, which was higher at the start of the study. This could be due to the effect of rumen development on plasma mineral concentration. Plasma Fe concentration was higher by the end of either studies which confirmed the biological availability of supplemental Fe.

Supplemental Fe in both studies reported herein had no effects on the Cp activity of lambs in experiment two, but there was a trend for the effect of Fe to reduce Cp activity in week 4. The result obtained in the current studies is in accordance with those observed in chapter 3 and those reported by Williams (2004). Breed of sheep also had no effect on Cp activity except in experiment one where Texel lambs had lower Cp concentration than Scottish Blackface in week 2. Data more relevant to animal nutrition has been indicated that Cp mRNA was not affected in rats that were exposed to deficient, normal, or high levels of dietary Fe (Tran *et al*., 2002). The activity of Cp has been found to decrease with Cu depletion in ruminants during primary Cu deficiency (Blakley and Hamilton, 1985), and also during the exposure to high dietary intakes of Mo (Humphries *et al*., 1983). It has been reported that Cp activity may increase as a result of infection, stress, or pregnancy (Linder, 1991). Therefore, Cp:Pl-Cu ratio was advocated to be a more accurate indicator than plasma Cp or Cp activity alone to assess the functional Cu status of ruminants (Mackenzie *et al.*, 1997). The theoretical ration of 2:1 (Cp:Pl-Cu) was proposed by Mackenzie *et al*. (1997) for normal ruminants, with a ratio <2.0 may show that TM was being absorbed into the blood, a ratio of 1.0-1.5 may indicate a definite TM problem and <1.0, a serious TM problem. This ratio was confirmed by Mackenzie *et al.* (2001). Kendall *et al*. (2001) reported an increase in the Cp:Pl-Cu ratio in cattle supplemented with Cu.

Effect of dietary Mo + S have consistently reduced the Cp:Pl-Cu ratio but dietary Fe has been in consistent with Williams (2004).

Multiple copper indices such as Cp and SOD activities and Cp:Pl-Cu ratio decreased with time in both of the studies which also in agreement with the results reported in Chapter 3 and 4. This reduction may not be of biological importance in the lambs and the reason for the decrease cannot be accounted for. WBC count increased in the group fed supplemented Fe, which is not in agreement with the result reported in Chapter 3 and 4, however was within the normal ranges reported in sheep.

5.5. Conclusions

High dietary Fe had no effect on sheep health and performance characteristics of lambs regardless breed. Results from the current studies indicate that high dietary Fe significantly reduces plasma Cu concentrations. Scottish Blackface had numerically higher plasma Cu concentration than Texels throughout the study which could be due to the higher Cp activity of Scottish Blackfaces as it counts for 80 – 94% of circulating Cu. Dietary Fe significantly reduced liver Mo concentration and increased plasma Mo and Fe in the second experiment. Inclusion of 368.7 mg Fe/kg DM was not enough to decrease liver Cu of growing sheep. Significant differences in Cu requirements were observed between Texel and Swaledale where 13.6 mg Cu/kg DM was not enough to meet Cu requirements of Swaledale lambs. Swaledale lambs were losing about 1.12 µg hepatic Cu per day. Dietary Fe had no effect on the liver minerals concentration of lambs except Mo which was reduced by Fe supplements. Swaledale lambs had a higher SOD activity than Texel lambs, but Fe had no effect on SOD activity of growing lambs. Iron supplements had no effects on Cp activity or Cp:Pl-Cu ratio than lambs given the control diet. The level of Fe supplementation was insufficient to elucidate a significant difference between the two breeds as the background level of Fe was high and supplementation of 368.7 mg Fe/kg DM was not enough to observe the significant effect on liver Cu concentration.

CHAPTER 6

General discussion

6.1. Introduction

Previous reports in the literature on the effect of Fe supplementation at different levels on performance, health, and digestibility in sheep and cattle have been unclear and inconsistent (Phillippo *et al*., 1987b; Williams, 2004). This makes determining the individual effect of Fe difficult to determine in sheep. Much of the focus of the Cu antagonists has been with Mo and S (Suttle,1975; Van Ryssen and Stielau, 1981; Allen and Gawthornet, 1987) with Fe being considered less significant. This has been reflected in much of the previous reports where Fe has usually only been used as a comparison as a single level. The objectives of the current series of studies were to investigate the effect of level of Fe with and without S on lambs performance and Cu metabolism and the effect of supplemental Fe on the Cu status of different breeds of growing lambs. The differences in the duration of each study reported in the current thesis was calculated by regression analysis utilising the data from Williams (2004) thesis in Chapter 1 and using the data from Chapter 1 to predict Cu depletion per day in Chapter 4 and 5 by feeding Fe supplemental diets to detect significant differences in the hepatic Cu concentration between lamb groups. Texel breed have been chosen as a model to investigate the effect of supplemental Fe on Cu metabolism in all experimental Chapters, as it is a known breed that is efficient in Cu absorption and retention and often suffer from Cu toxicity.

In the first experiment (reported in Chapter 3), four treatments of different levels of dietary Fe at constant level of S were fed to determine the effect of Fe on Cu metabolism of lambs. It was found that Fe supplementation had no effect on lamb performance but significantly reduced liver Cu storage, but increased liver Fe concentration in a dose response manner concurring with the results of other researcher in sheep and cattle (Standish *et al*., 1969; Bremner *et al*., 1987; Prabowo *et al*., 1988). The second experiment reported in Chapter 3 dietary Fe had no effect on nutrient digestibility. Following on from the results obtained from these two experiments, work was then conducted to examine the effect of S with or without Fe on Cu metabolism and performance of lambs. In this experiment six treatments were used; three levels of dietary S and two levels of dietary Fe. The results indicated that Fe was a potent Cu antagonist in weaned lambs and that it is effect was independent of dietary S supply. Fe supplementation reduced liver and plasma Cu concentration of the growing lambs independently of S level in the diet. A positive relationship of FeS was observed to

increase plasma and biliary Mo concentration in the lambs fed H:H diet present in Chapter 4. Work was then conducted to investigate the effect of breed and Fe on Cu metabolism and performance of lambs in two experiments. In the first experiment 2 levels of added dietary Fe (0 and 800 mg Fe/kg DM) and two breeds of lambs (Texel and Scottish Blackface) were used. In experiment 2, two levels of added dietary Fe were also used but lower than the first study (0 and 368.7 mg Fe/kg DM), were given to Swaledale and Texel lambs. In experiment one an additional 800 mg Fe/kg DM reduced plasma Cu concentration of both breeds from week 2 to the end of the study. Scottish Blackface lambs had a higher plasma Cu concentration than Texel lambs. Plasma Mo increased in the groups given supplemental Fe and Texel lambs had a higher plasma Mo concentration compared with Scottish Blackface lambs fed the same diets which could be due to the suggested interaction between Fe and S in the intestine that gives more chance for Mo absorption or high dietary Fe may increase Mo depletion from the body tissue and increase Mo concentration of the urine. In experiment 2, supplemental Fe had no effect on liver Cu status of both breeds of lamb, but reduced plasma Cu of the lambs although no difference between breeds observed. Texel lambs retained higher liver Cu than Swaledale lambs; however, Swaledale lambs had a higher initial liver Cu concentration than Texel lambs. The chemical analysis of the basal diets used in this series of studies would be important to discuss due to the dietary mineral concentration of the diets given to animals in Chapters 3 to 5 (Table 6.1). The mineral composition of the basal diet present in Table 6.1 indicates that Cu concentration of the diets was slightly higher than expected (MAFF, 1992) especially in Chapters 3 and 5 (experiment 2). Therefore, this may count for the lack of the effect of Fe on plasma and liver Cu concentration as described in chapter 5. Fe concentration of the basal diets used in Chapters 3 and 5 (experiment 2) was higher than the predicted values from MAFF (1992) which may have had an effect on the parameters studied in both studies and could be behind the lack of the effect of treatments on Cu statue of lambs however, little information is available on the bioavailability of Fe from dietary sources. The Mn concentration of the basal diet used in Chapter 1 was higher than its concentration in Chapters 4 and 5, however was not as high as the concentration that might affect Cu metabolism the lambs (NRC, 2005). Mo concentration of the basal diet used in Chapter 3 (experiment 2) was higher than the concentration predicted however, no effect on lamb health was observed through the study and no effects were observed on plasma Cu of lambs. High Mo concentrations of the diets in the presence high S have been reported to increase plasma Cu concentration of lambs (Williams, 2004) and increase plasma Mo concentration due to the formation of TM and increasing the concentration of unavailable Cu in the plasma. However, in the current studies there was no evidence of TM formation. Mo concentration of other diets was within the normal ranges and reported in the animal diets and it is unlikely to affect Cu metabolism or to produce TM. The S concentration of

the basal diet of chapter 4 was high as the experiment was designed to provide 4 g S/kg DM and its concentration was within normal range in chapter 4 and 5 (experiment 2) which is unlikely to affect Cu status of lambs as Mo level of the diets was low. However, S concentration of the basal diet used in chapter 5 (experiment 1) was higher than expected. Suttle (1974d) reported than dietary S intakes >2 g/kg DM reduced Cu availability in sheep, but Grace (1997) did not find similar effects in sheep. Zinc concentration of the all diets was normal and it is unlikely to have affected Cu metabolism of the lambs.

Table 6.1. Mineral composition of the basal diets (mg/kg DM) – Chapter 3 to 5 inclusive

Item	Chapter 3		Chapter 4	Chapter 5	
	Expt. 1	Expt. 2		Expt. 1	Expt. 2
Cu	9.85	11.1	9.51	8.2	13.6
Fe	487.6	1086.6	302.3	257.5	532.4
Mn	137.5	116.8	72.4	58.1	78.1
Mo	2.5	4	1.2	1.7	2.1
S (g/kg DM)	4.2	4.2	2.9	4.8	2.9
Zn	97.8	83.5	78.7	66.8	83.8

6.2. Effects of iron on performance

There was no clinical sign of Cu deficiency observed throughout all experiment reported in this thesis. Plasma and liver Cu concentration were within normal ranges (Table 6.2). Besides the results from all chapters indicated no significant effects of Fe supplementation (from 250 to 800 mg/kg DM) on performance parameters such as DM intake, DM digestibility, DLWG and FCR of growing lambs. These results are in agreement with the results reported by many researchers in sheep (Prabowo *et al*., 1988; Williams, 2004) and cattle (Phillippo *et al*., 1987a). However, other research in cattle observed that high intake of Fe (73.5 g/d) reduced LW and caused scouring (Coup and Campbell, 1964) (Table 6.3). Coup and Campbell (1964) also observed a decrease in milk yield and herbage digestibility when dairy cows were given 30 g Fe/d. Standish *et al.* (1969; 1971) found a significant decrease in the feed intake and DLWG by feeding cows a diet supplemented with 500 mg Fe/kg DM.

For the performance of a group of lambs that are of similar genetic background and balanced for sex to be affected there must be an alteration in either nutrient supply of nutrient utilisation/metabolism (Lawrence and Fowler, 2002). All trials used lambs that were fed at a restricted level and there was no effect on DMI so effects on appetite may be missed.

Table 6.2. Liver and plasma copper concentration of the lambs

Chapter	Fe[1]	Initial Cu concentration		Final Cu concentration	
		Liver (mg/kg DM)	Plasma (µmol/l)	Liver (mg/kg DM)	Plasma (µmol/l)
3	0	313.6	13.7	313.3	11.6
	250			322.3	11.5
	500			242.8	11.5
	750			205.0	10.5
4	0	591.1	15.5	545	12.1
	800			274	10.4
5[2]	0	(265; 159)	(20.1; 23.7)	191; 294	(15.7; 13.1)
	368.7				(13.1; 13.3)

[1]dietary supplemental Fe (mg/kg DM)
[2](Swaledal; Texel)

All lambs started their studies with normal to high liver and plasma Cu values (Table 6.2). Also at the completion of the trials, although there was usually a decrease in Cu status, lambs were within the range considered as adequate (Table 6.2).

A Cu dependent enzyme, PAM (peptidylglycine α-amidating monooxygenase) is known to affect the secretion of several hormones that affect appetite such as gastrin and leptin. Although no studies have shown copper affecting the release of these in ovine animals, another PAM dependent hormone, ACTH was shown to accumulate in tissue and hence dietary Mo but not Fe (Williams, 2004) inhibited its release. The other aspect of nutrient supply is nutrient digestibility. This was shown not to be affected by dietary Fe (Chapter 2). Performance can be affected by nutrient utilisation, for example, a reduction in the enzyme cytochrome C oxidase has been linked with alteration in energy capture from diet (Michels *et al*., 1979). This was not measured in this study, but from previous work, there have been no reports of high Fe supply inhibiting the activity of this enzyme.

Previous studies on the effects of Fe on animal performance have been inconsistent and have been summarised in Table 6.3. Levels of supplementation where Fe has reduced performance are generally much higher than those used in this series of studies. For example, Koong *et al*. (1970) found no reduction in ADG of calves given diets containing high levels of Fe ranging from 100, 500, 1000 to 2000 mg Fe/kg DM, (1.27, 1.23, 1.17 and 1.13 kg/d respectively), but a significant reduction in DLWG were observed when total Fe content of the diet was increased to 2500 or 4000 mg Fe/kg DM (0.85 and 0.65 kg/d, respectively). Koong *et al*. (1970) were also found a reduction in daily DMI in groups fed 2500 and 4000 mg Fe/kg DM (4.35 and 4.02 kg/d, respectively) compared with those fed 100 and 1000 mg Fe/kg DM (5.13 and 4.05 kg/d, respectively). Similar reductions in DLWG and DM intake was observed by Standish *et al*. (1969) in steers given a diet

supplemented with 1600 mg Fe/kg DM compared to those given 400 mg Fe/kg DM (0.39 vs. 0.63 kg/d) and the FCR of cattle when fed a diet supplemented with 4000 mg Fe/kg DM. Whitelaw *et al*. (1979) found a significant difference in LWG of Scottish Blackface lambs (2.5 kg after 12 weeks) when they were grazing an improved hill pasture. The cause behind that decrease in LWG was suggested to be due to the effect of molybdenum-sulphur that induced Cu deficiency compared with those given Cu injections. Similarly, Woolliams *et al*. (1986) when using two lines of lambs that had been selected on the bases of plasma Cu concentration (L and H) from pure Scottish Blackface and Welsh Mountain breeds, found that the lambs with H plasma Cu concentration were always heavier and were slaughtered before the lambs having L plasma Cu.

Table 6.3. Review table of the effect of iron on animal performance and copper metabolism

Iron level	Performance	Liver (mg/kg DM)	Plasma (µmol/l)	Reference
30 mg/kg LW	No effect	7	5.19	(Campbell *et al.*, 1974)
30 g/d	Decrease milk yield and digestibility	-	-	(Coup and Campbell, 1964)
60 g/d	Lowered butterfat, losses of LW	-	-	
100	No effects	-	15.74[1]	(Koong *et al*., 1970)
500			15.74	
1000 2000			17.31	
100	Reduced DLWG, DM intake, FCR	-	15.74	(Koong et al., 1970)
1000			17.31[1]	
2500			15.74	
4000			17.31	
0	Loss of BW	678	15.74	(Standish and Ammerman, 1971)
1,600 ($FeSO_4$)		528	16.36	
1,600 ($C_6H_5FeO_7$)		656	11.17	
0	Reduced DM intake, DWG, FCR	260	17.46	(Standish *et al.*, 1969)
400		145	20.92	
1600		44	21.55	
3200		-	20.30	
100	Reduced DLWG, DM intake	177	-	(Standish *et al.*, 1971)
1000		50	16.84	
145[2]	No effect on the growth	343	15.42	(Bremner *et al*., 1987)
730		374	11.0	
1460		388	12.2	
0	No effect on performance	110	12.0	(Humphries *et al*., 1983)
800		94.5	12.27	
0	No effect on performance	52.9	11.80	(Phillippo *et al*., 1987a)
500		5.5	11.49	
0	No effect on performance	297	2.68	(Prabowo *et al.*, 1988)
300		270	13.3	
600		242	13.1	
1200		186	11.5	
			10.2	

[1]Serum Cu
[2]pre-ruminants

6.3. Plasma Copper

Currently plasma Cu concentration is the most commonly used diagnostic indicator of Cu status of animal because it is convenient to collect and separate and because it can be used for multiple laboratory analyses (Herdt and Hoff, 2011). However, plasma Cu levels alone are believed to not be an accurate indicator of Cu status of animals (Herdt *et al*., 2000; Kincaid, 2000; Herdt and Hoff, 2011). Normally it is accepted that a low plasma Cu concentration (<7.9 µmol/l) would indicate a low liver Cu store (Claypool *et al.,* 1975). Plasma Cu concentrations from 12 – 24 µmol/l (Herdt and Hoff, 2011) or 9.4 – 19 µmol/l (Rushton, 1981) is considered the normal ranges in sheep and cattle. However, depending on plasma Cu concentration alone to assess Cu status of animal may not fully represent Cu status of sheep as plasma Cu may stay high when TM inters the blood although that Cu is unavailable for the animal use (Dick *et al.*, 1975; Williams, 2004). The plasma Cu concentration of the lambs used in experiment 2 (presented in Chapter 5) was over 20 µmol/l at the start of the study, although liver Cu concentration was within the normal ranges reported for ruminants (NRC, 2005) (Table 6.2), while the plasma Cu concentration was 17.21 µmol/l when the liver Cu concentration was just above 300 mg/kg DM.

In the series of experiments reported in this thesis, it was observed that dietary Fe had no consistent effects on the plasma Cu concentration. For example: in Chapter 3, the lambs given dietary Fe supplements of 250, 500, or 750 mg/kg DM had no effect on plasma Cu concentration up to 6 weeks. In the experiment reported in Chapter 4, feeding 800 mg supplemental Fe/kg DM significantly reduced plasma Cu concentration at week 12. Using a similar level of supplemental Fe (800mg/kg DM) in another experiment reported in Chapter 5 (Experiment 1) showed a similar decrease from week 2 to the end of the study. This is despite the hepatic Cu concentration of the lambs being in the normal range. Therefore, Fe supplements did not have consistent effects on the plasma Cu concentration of lambs. It appears that significant changes in the plasma Cu of the lambs given supplemental Fe can be observed quicker when the initial plasma Cu level was high (>16 µmol/l). In contrast, when the plasma Cu concentration was low at the start of the study (<16 µmol/l) and liver Cu concentration higher than 300 mg/kg DM, significant changes in the plasma Cu can be observed late (e.g. about 12 weeks). The minor effect of supplementary Fe on the plasma Cu concentration of growing lambs observed in Chapter 5 after week 5 (Experiment 2) is unclear. Plasma Cu can increase at the times of stress such as shearing, weather extremes, and transport or during an acute phase reaction as a result of infection or inflammation (Jones and Van Der Merwe, 2008). None of these events was observed in the lambs in the current studies.

Terada *et al*. (1995) demonstrated that 90 – 95% of plasma Cu is in Cp that is synthesised and secreted by the liver. Dowdy and Matrone (1968) and Suttle (1974) have reported that high dietary Mo reduced Cu uptake in the small intestine. Nevertheless, other researchers found increases in the plasma Cu with increasing dietary Mo (Dick *et al.*, 1975; Williams, 2004). Hence, plasma Cu concentration does not reflect the actual Cu status of sheep. Wentink *et al*. (1999) suggested that depending on the blood Cu concentration alone to assess Cu status of sheep may mislead researchers and may not truly represent Cu status of animal. Plasma Cu concentrations alone should be monitored with caution; the Cu bound to TM may not be available (Price *et al*., 1987) but still can be measured in the plasma which can give diagnostic reading which is within the normal known ranges of ruminants. The TCA-soluble Cu which is proposed by Tompsett (1934), is usually used to measure the available plasma Cu of ruminants. From the TCA-soluble Cu results (present in Chapters 3 and 4), it is clear that there was no evidence of TM in the blood. However, there was a trend in the lambs given high S supplements in Chapter 4 to have lower plasma TCA-soluble Cu by the conclusion of the study.

Supplemental Fe increased plasma Mo concentration of the lambs in Chapters 3, 4, and 5. Similar increases in the plasma Mo concentration were also observed in cattle given a diet supplemented with 800 mg Fe/kg DM; however the researchers did not suggest a mechanism for increases observed in the plasma Mo concentration of the group fed high Fe diet (Humphries et al., 1983). Gould and Kendall (2011) extensively reviewed the effect of high Mo and S on Cu absorption and metabolism and Mo, S and Cu interactions in the rumen. The authors reported that Mo in the rumen react with S to produce different TM classes (Figure 6.1) that react with Cu and reduce Cu absorption. If Cu concentration is low in the rumen, TM can enter the general circulation through rumen walls and reduce the activity of Cu containing enzymes to produce a systemic effect. From the experiments described in this thesis, it is assumed that there was no evidence of TM production as TCA-Cu soluble and CP:Pl-Cu ratio were not significantly altered. Therefore, the high plasma Mo concentration observed in the lambs give Fe supplements could have been due to effect of Fe on Mo absorption from the diet or to the effect of Fe on mobilisation of Mo from the storage organs.

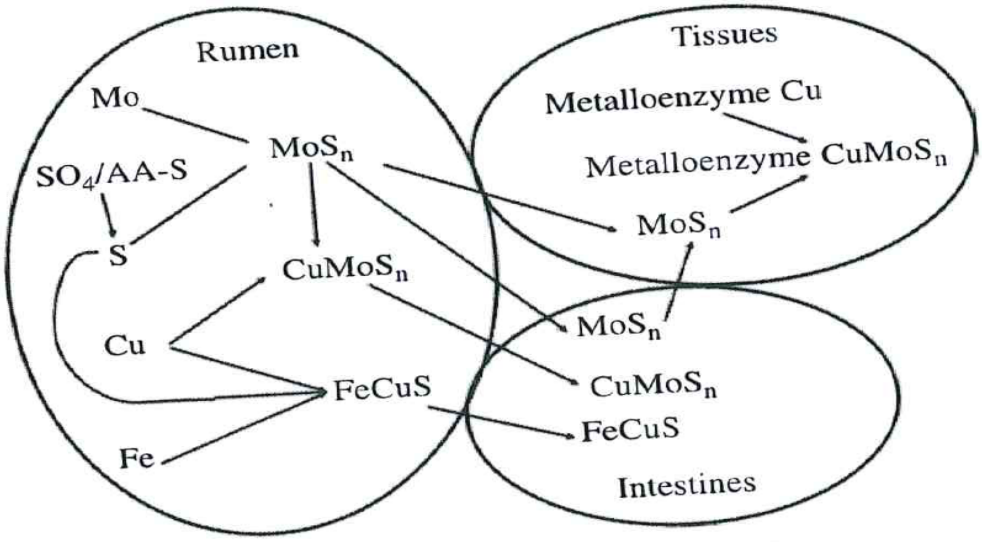

Figure 6.1. Ruminal mechanism for the interaction of Cu, Mo, S and Fe and routes of absorption for the interaction products

Generally, all plasma minerals were observed to decrease with the progress of the studies except plasma Fe that increasing in the groups fed Fe supplemental diets. These changes in the level of plasma minerals with time were also observed by Williams (2004) in studies with lambs. It is suggested that the lambs used in the current theses mostly were young in their age (3 – 4 months) and it is well known that young lambs are very efficient at mineral absorption and retention. Therefore, with the progress of each study lambs were grown more and their rumen was developing which is a very good environment for microorganisms growth which in turn can affect mineral availability and reduce their absorption.

6.4. Liver Copper status

The liver has a capacity to accumulate Cu and maintain plasma Cu concentration within the normal ranges especially in simple stomach species unlike ruminants as they did not develop an efficient mechanism to excrete an extra amounts of liver Cu through bile (Suttle, 2010; Herdt and Hoff, 2011). The liver represents the main storage pool of Cu in the body and it reflects the long-term dietary Cu supply (Herdt and Hoff, 2011). Liver Cu depletion is the earliest sign of Cu deficiency in ruminants. Consequently, liver biopsy samples are the most sensitive and accurate mean of evaluating dietary Cu supply in ruminant (Herdt and Hoff, 2011). Copper bioavailability is influenced by the concentration of other trace elements, especially Mo and S (Herdt and Hoff, 2011) and Fe (Arredondo *et al.*, 2003; Garrick *et al.*, 2006). At hepatic Cu concentrations of >30 - 50 mg/kg DW, there is a little correlation between liver and blood Cu concentration (Herdt and Hoff, 2011). Deficient blood Cu values are generally not observed until liver Cu concentration decrease

to less than approximately 25 mg/kg DW (Claypool *et al.*, 1975; Mulryan and Mason, 1992). However, even at low hepatic Cu concentration, the relationship between blood to liver Cu values is inconsistent (Herdt and Hoff, 2011). Some animals with normal plasma Cu level may have low liver Cu concentration. Therefore, relying on the plasma Cu concentration alone to estimate Cu status of animal may not reflect the actual Cu status of animal.

In the experiments reported in Chapters 3 and 4, supplemental Fe (>500 mg/kg DM) reduced liver Cu concentration compared with the lambs given no Fe supplements. This is in accordance with the results reported by other researcher in sheep (Prabowo et al., 1988; Williams, 2004) and cattle (Bremner *et al*., 1987; Humphries *et al*., 1983; Phillippo *et al.*, 1987a,b). The relationship between the level of Fe supplementation and liver Cu concentration of the lambs used in this thesis is present in Figure 6.2. Sulphur supplementation as a main effect or interaction had a minor effect on the liver and plasma Cu concentration of the lambs and had no additive effects on the liver Cu retention of lambs given Fe supplemental diet (see Chapter 4). The result observed in Chapter 4 does not agree with those reported by Suttle *et al*. (1984) in sheep and Bremner *et al*. (1987) in calves, who suggested that S is crucial in reducing Cu absorption of calves through a direct effect on Cu in the rumen. Suttle *et al*. (1984) reported that S was important to the inhibitory effects of ingested soils high in Fe concentration on Cu absorption of sheep. Suttle *et al*. (1984) have suggested that the soil constituent most likely to react with S is Fe. Suttle *et al*. (1984) assumed that Fe salts might inhibit intestinal Cu absorption by a temporarily trapping, as FeS, sulphide that is normally absorbed rapidly from the rumen. The trapped sulphide is believed to be released in the acid environment of the abomasum to react thereafter with Cu forming insoluble and unavailable CuS. Hepatic Cu concentration of the lambs used in the experiments reported in this thesis (reported in Chapter 3 to 5) did not reach the level which is indicative of clinical deficiency Cu (<100 mg/kg DM) by the end of each study. These findings are in accordance with those reported by Prabowo *et al.* (1988) and Williams (2004) in sheep and Phillippo *et al*. (1987b) and Humphries *et al*. (1983) in heifers.

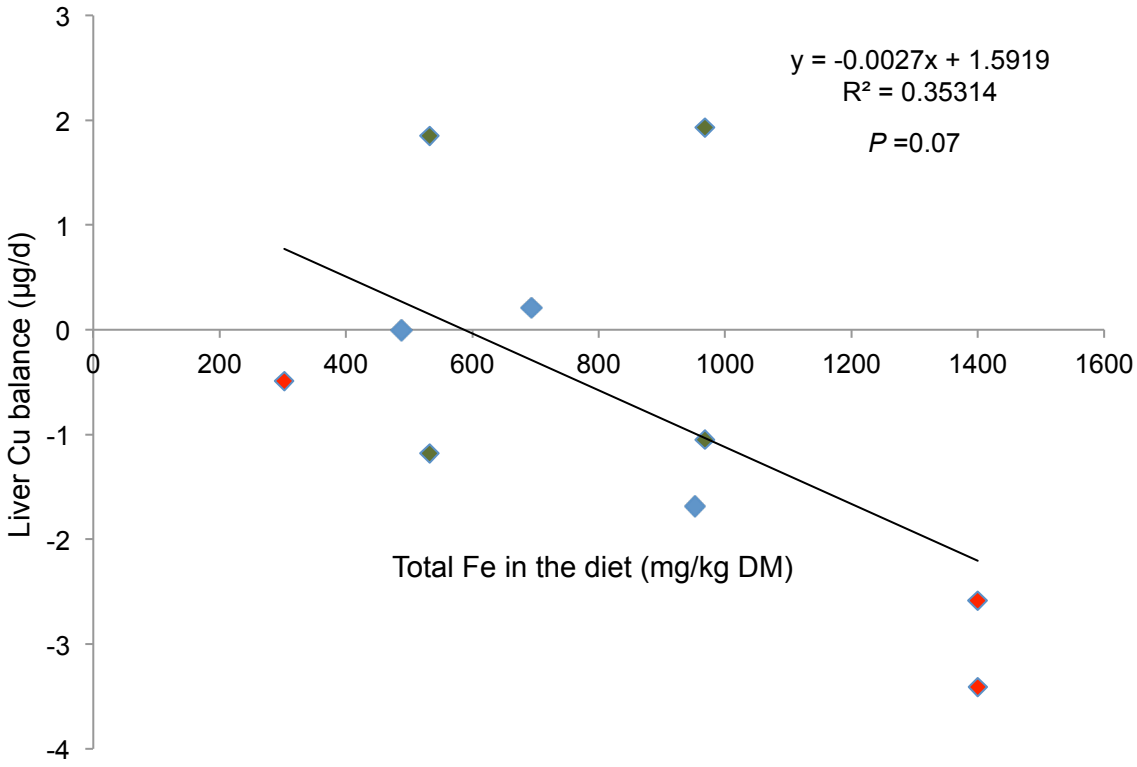

Figure 6.2. Effect of the total dietary iron on liver copper balance of growing lambs (µg/d), Blue symbols are the data from Chapter 1, red are data from Chapter 4, and green are from Chapter 5.

Yu *et al.* (1994) conducted a 6-week study to investigate the effect of supplemental Fe (low: 7, normal: 40, or high: 389 mg Fe/kg DM) on Cu homeostasis of rats. Yu *et al.* (1994) observed a disturbance in the Cu homeostasis of the rats similar to those found in swine; supplemental Fe resulted in a linear decrease in the apparent Cu absorption and biliary Cu excretion. Previous data in cattle has showed that high dietary Fe concentrations reduced apparent Cu absorption (Standish *et al*., 1971), this may explain the liver Cu reduction of sheep fed high dietary Fe reported in Chapter 3 and 4.

Humphries *et al*. (1983) demonstrated that supplemental Fe (800 mg/kg DM) reduced liver Cu by 95%, plasma Cu to <9 µmol/l and SOD activity by 66% in weaned Hereford-Friesian and Aberdeen Angus heifers after 32 weeks compared to controls. Gengelbach *et al.* (1994) also found that dietary Fe supplementation (600 mg Fe/kg DM) reduced plasma Cu and Cp in heifers and their offspring. Fry (2011) demonstrated that high dietary Fe (750 mg/kg DM) increased liver Cu efflux of cattle. There are two mechanism by which Fe could have the most dramatic effect on Cu metabolism, at the site of absorption in the small intestine or increase Cu efflux from the liver (Humphries *et al*., 1983; Yu *et al*., 1994).

High dietary Fe decreased apparent Cu absorption in sheep (Suttle and Peter, 1985) and rats (Yu et al., 1994). Using a molecular approach, Lee *et al*. (2002) demonstrated that Hek-293 cells (Human embryonic kidney cells 293) expressing with human CTR1 had lower ^{64}Cu uptake when incubated with 50 molar excess of Fe. Lee *et al*. (2002) suggested that high Fe may be a reversible inhibitor of the human CTR1 protein and with increasing Fe concentration of the media, CTR1 activity may go down.

Fry (2011) found that dietary supplemental Fe (750 mg/kg DM) did not affect duodenal or hepatic CTR1 and mRNA expression of calves. Fry (2011) suggested that the lack of effect of Fe on mRNA and CTR1 expression could be due to the high dietary Fe concentration. Approximately a 2 fold increase in mRNA expression of the hepatic Cu efflux pump, ATP7b was observed in calves fed 750 mg Fe/kg DM which likely explains the decrease in the liver Cu storage (Fry, 2011). This hepatic Cu exporter (ATP7b) is responsible for biliary Cu excretion and incorporation of Cu into the Cp (Kim *et al*., 2008). Recently it has been documented that high dietary Fe increases porcine ATP7b mRNA with associated increases in biliary Cu concentration (Fry *et al.*, 2010). If increased expression happened in this series of studies then it may explain why there was a decrease in liver Cu along with an increase in Cp activity.

6.5. Plasma enzymes

There are a number of enzymes that contain Cu in their structure and are used to indicate the Cu status of animal. Copper containing enzymes includes Cp and SOD (Suttle, 2010). Ceruloplasmin have an oxidase activity which have first described by Holmberg and Laurell (1948). It was found in the plasma of most species and is synthesised in the liver (Gitlin and Schroeder, 1992). In sheep and cattle, the amount of Cu per protein molecule vary from 6 – 8 atom/molecule (Calabresel *et al.*, 1983). Ceruloplasmin is a poly functional protein which is involved in a free radical scavenging and anti-oxidant activity (Goldstein and Charo, 1982), Cu and Fe transport (Linder and Hazegh-Azam, 1996), and it also incorporate Fe into the storage protein, ferritin (Saenko *et al.*, 1994). It is an important acute-phase reactant involved with inflammation (Linder and Hazegh-Azam, 1996) that maybe used as an indicator of Cu status of sheep, and Cp activity reported for sheep ranges from 12 – 24 mg/dl. Adequate Cu is essential to proper Fe metabolism, as Cu-dependent ferroxidases are necessary for mobilized Fe out of tissues to the sites of RBC formation (Osaki *et al*., 1971).

The results reported in Chapter 3 to 5, showed that Cp activity was within the normal range reported in sheep (12 - 24 mg/dl) at the start of all studies. Although the Texel lambs used in Chapter 5 (Experiment 2) had a higher Cp activity than the normal range reported for sheep (29.51 mg/dl) when reduced to normal ranges after one week after the

start of the study. This could have been due to the effect of stress during transport as the lambs were sourced from elsewhere.

The effect of dietary treatment and breed differences on the activity of Cp were obvious in Chapter 5 (Experiment 2) where Texel lambs given Fe supplemental diet had a higher Cp activity compared with those given no Fe supplemental diet. In all studies reported in Chapters 3 to 5, Texel lambs given the Fe supplemented diet had a higher Cp activity than Texel lambs given no Fe supplemental diets. In contrast, Scottish Blackface and Swaledale lambs fed Fe supplemented diet had a lower Cp activity than those given no Fe supplemented diet. In most studies reported in sheep and cattle, high dietary Fe reduced Cp activity of animals while the current results are different and showed that Texel lambs fed Fe supplements had an increased Cp activity than those fed no Fe supplemental diets, but opposite results were observed in Swaledale and Scottish Blackface lambs given an Fe supplemented diet. Similar increases in Cp activity were also observed by Fry (2011) in weaned calves give diets supplemented with 750 mg Fe/kg DM. Fry (2011) found approximately a 2-fold increase in the mRNA expression of the hepatic Cu efflux pump (ATP7b) in high Fe fed calves. This hepatic Cu exporter (ATP7b) is responsible for biliary Cu excretion and incorporation of Cu into Cp (Kim *et al*., 2008). Humphries *et al*. (1983) found a significant reduction in Cp activity of weaned calves given diets supplemented with 800 mg Fe/kg, 5 mg Mo/kg DM or a combination of both Fe and Mo. Rapid decreases in the Cp activity of sheep was observed when given a duodenal Mo infusion (Mason, 1982). Gooneratne *et al*. (1981) suggested that plasma Cp activity decreases after a prolonged exposure to TM. The differences observed in the Cp activity between the current results and other researcher results may have been due to the different effects of Fe supplements on mechanism of Cp production among different breeds of sheep used in this thesis. With the progress of experiment in all experiments reported in this thesis, Cp activity was reduced although did not reach the suggesting deficiency level (<8 µmol/l) by the end of all studies but it was always higher in Texel lambs fed Fe supplemental diets and Scottish Blackface and Swaledale lambs fed control diet. Similar reductions were also observed by Williams (2004).

Approximately 60% of Cu in the erythrocytes is associated with SOD (Kincaid, 2000). However, SOD activity is not a sensitive measure of Cu status and does not drop with deficient intakes of Cu until after plasma Cu and Cp are reduced (Andrewartha and Caple, 1980; Ward and Spears, 1997; Gengelbach and Spears, 1998). Superoxide dismutase is the first line of defence against reactive oxygen species and is active in catalysing detoxification of superoxide radicals (Gonzales *et al*., 1984). SOD catalyses the dismutation of the superoxide radical to hydrogen peroxide and water (Nazifi *et al*., 2010). In Chapter 4, dietary Fe supplements reduced the SOD activity of lambs in week 12.

While in Chapter 3, dietary Fe had no effects on SOD activity of lambs, that could have been due to the short period of the study. Williams (2004) also did not found significant effects of supplementing 500 mg Fe/kg DM, for 10 weeks on SOD activity of lambs, but those given 5 or 10 mg Mo/kg DM had significantly lower SOD activity than control or Fe supplemented groups in week 9 of the study. Sheep are unique at having two populations of red cells, one short-lived (70 days), the other long-lived (150 days) (Kerr, 2002). Therefore, to observe the effect of Fe supplements on SOD, longer time periods may have been required. Similarly, Bremner *et al.* (1987) did not find significant effects of Fe supplementation on SOD activity of weaned calves. SOD activity of heifer claves decreased by 50% after 16 weeks and further reduction were recorded from 16 to 32 weeks when given a diet supplemented with 800 mg Fe/kg DM (Humphries *et al*., 1983). It has been found that erythrocyte SOD activity increased in response to Cu supplements in sheep (Andrewartha and Caple, 1980) and cattle (Phillippo *et al*., 1982).

6.6. Conclusion and further work

From the series of the studies reported in this thesis, some novel findings were observed with respect to the antagonistic effect of supplemental Fe with or without S on liver and plasma Cu concentration and on the serum Cp activity. Reductions in the liver or plasma Cu concentration were confirmed in the experiments reported in this thesis. Minor effects of supplemental Fe on SOD activity were observed in this series of studies and increases in the Cp activity of lambs were observed in the lambs given high Fe supplemented diets. Breed differences in Cu metabolism were clearly observed which confirm the findings of other authors. In this thesis, the effect of Fe supplementation on Cp activity of different breeds of sheep was observed which has not reported previously in sheep. It was also concluded that Fe supplementation increased plasma Mo concentration of lambs and decreased liver Mo concentration that may have an effect on plasma and liver Cu concentration of lambs.

The precise mechanism behind the effect of dietary Fe level on Cu status of sheep have not been achieved in this thesis and further work is required to fully understand the reduction in the plasma and Cu concentration of lambs fed high Fe containing diets. Supplemental Fe in the current thesis had no effects on the lamb performance and the lambs showed no clinical sign of Cu deficiency throughout the period of the studies which may have been due to the restricted feeding. Therefore, Fe supplementation (at certain levels) could be used in sheep diets which are prone to Cu toxicity without showing any side effects on performance or health. The use of Fe to prevent hepatic toxicity in chronic and acute Cu overloaded sheep would be of great interest to UK agriculture and should form the basis of future work.

Further work will be beneficial to investigate the effect of Fe supplementation on Mo metabolism of sheep and related to Cu metabolism in addition to study the effect of Fe supplementation on the proteins important in Cu transported throughout the body and within a cell (Cu trafficking). Differences between breeds in term of Cu absorption and retention and effect of Fe on Cu metabolism of different breeds of sheep also needs to be investigated more intensively using qPCR and the differences in the expression of different Cu chaperons needs to be included.

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