The Copper Conundrum:

Elucidating the Modes of Copper Antagonism in the Rumen

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I dedicate this work to my husband Phil,

without whose love and support I would have never have begun this journey.

"Are you sitting comfortably? Good. Then I'll begin..."

Abstract

Copper is an essential trace element. In ruminants interactions occur during digestion which can interfere with copper absorption. Most notably, both iron and molybdenum in combination with sulphur. If there is insufficient copper available, thiomolybdate, formed from molybdenum and sulphur, can be absorbed and subsequently interfere with systemic copper function. It has not yet been established if copper-thiomolybdate is able to persist in the liver, or if the different copper sources participate differently in these interactions. The mode of action of the iron-sulphur pathway has also not been elucidated and very little is known about the nature of this interaction.

The aim of this work was to investigate the interactions of copper and its antagonists for a range of copper sources at different regions of the ruminant digestive tract. To elucidate the mode of iron-sulphur antagonism and to investigate the relationship between antagonist routes. A secondary aim was to assess the understanding of copper-related terminology and problems, across the farming community through a national survey.

The survey demonstrated that copper-related knowledge was not well understood across the farming community and copper supplementation is not as targeted as it could be. Initial *in vitro* work demonstrated that simulation of rumen conditions was not sufficient for investigation of the iron-sulphur pathway and that the solubility of different copper compounds was similar, but not directly comparable to bioavailability. This lead to *in vivo* work which demonstrated the potency of thiomolybdate as a copper inhibitor and suggested that iron may have two modes of action; a soluble, un-absorbable compound and an insoluble compound. This work also discovered the omasum as a region of copper accumulation. X-ray absorption studies revealed the ability of the rumen to reduce copper into monovalent and elemental copper, and potentially form a complex with sulphur, iron, oxygen and chloride in the jejunum. Further *in vivo* work showed that none of the copper sources were able to prevent molybdenum absorption or to replete blood parameters. However, tribasic copper chloride was the most resistant to antagonist challenge, but its solubilisation in the rumen and omasal-flow differed to its hypothesised mode of action. Analysis of bovine and ovine livers using X-ray absorption indicated the presence of copper bound to metallothionein, glutathione and possibility of thiomolybdate presence in high copper status livers.

This work has shown that the iron-sulphur antagonism may be more complex than previously believed and that the rumen, omasum and jejunum are all potential regions where changes can occur to copper complexation. Different copper sources may be able to negate some of the antagonist effects, but not entirely, and require further investigation.

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Glossary of Terms & Definitions

General abbreviations

ACAF	Advisory Committee on Animal Feedingstuffs
AHVLA	Animal Health Veterinary Laboratories Agency
BW	Body weight
Cp:Cu	Caeruloplasmin to plasma copper ratio
DM	Dry matter
DMI	Dry matter intake
EDTA	Ethylene-diamine-tetra-acetic acid
EPR	Electron Paramagnetic Resonance
Hb	Haemoglobin
HSAB	Hard-Soft Acid-Base theory
ICP-MS	Inductively coupled plasma- mass spectrometer
LEC	Long-Evans Cinnamon rats
LH	Lithium heparin
MPL	Maximum permitted level
NRC	National Research Council
PEG	Polyethylene glycol
redox	Oxidation-reduction
ROS	Reactive oxygen species
TBCC	Tribasic copper chloride
TCA	Trichloroacetic acid
VFA	Volatile fatty acid
WW	Wet weight
XRD	X-ray diffraction
$\tilde{x}$	Median
$\bar{x}$	Mean
±	Standard deviation

Chaperones, transporters and enzymes

ALP	Alkaline phosphatase
Atx1	Antioxidant 1
CCO	Cytochrome c oxidase
Cp	Caeruloplasmin
Ctr1	Copper transporter 1
DMT1	Divalent metal transporter 1
MTF1	Metal transcription factor 1
SOD	Superoxide dismutase

Definitions

Antagonist	Dietary elements which are able to interact or bind with copper inhibiting copper uptake or bioactivity
Anoxic	Without oxygen
Bioavailable	A form able to be absorbed and utilised by the body
Copper-imbalance	A copper status or supply that fails to provide, or exceeds, optimal
Copper-loaded	Animals with a liver copper status above normal range
Copper lock-up	Labile copper has become insoluble through complexation with dietary antagonists
Labile	Easily broken down or displaced
Lixiviation	The process where soluble and insoluble substances are separated by dissolving the soluble component in water
Molybdenosis	Clinical condition arising from excess molybdenum
Molybdenum	Chemical element with the symbol Mo and atomic number 42
Oxic	Oxygen containing
Primary deficiency	Insufficient copper intake to meet requirements

Secondary deficiency	A condition where adequate copper supply is interrupted by copper antagonists
Thiomolybdate	A molybdate anion containing sulphur atoms
Thiomolybdate toxicity	Clinical condition arising from the absorption of thiomolybdate which subsequently interferes with biological copper

X-ray Absorption Spectroscopy terminology & XAFS equation parameters

µE	Spectra in energy
Å	Angstrom
E^0	Absorption energy
eV	Electron volt
FEFF	Effective scattering factor
K^2 (K-space)	Weighted photoelectron wave oscillation
LCF	Linear combination fitting
PCA	Principle component analysis
R-space	Radial structure function (Fourier transformed)
XAFS	X-ray absorption fine structure
XANES	X-ray absorption near edge spectra
XAS	X-ray absorption spectroscopy
σ^2	Debye–Waller factor

The XAFS equation: $\chi(\kappa) = S_0^2 \sum_i N_i \frac{f_i(k)}{kD_i^2} e^{-\frac{2D_i}{\lambda(k)}} e^{-2k^2\sigma_i^2} \sin\left(2kD_i + \delta_i(k)\right)$

Theoretically calculated values:

$f_i(k)$	Effective scattering amplitude
$\delta_i(k)$	Effective scattering phase shift
$\lambda(k)$	Mean free path
D_i	Initial path length

Parameters determined from fit to data:

N_i	Degeneracy of the path
S_0^2	Passive electron reduction factor
σ_i^2	Mean squared displacement
E_0	Energy shift extrapolated from k^2
ΔR	Change in half-path length extrapolated from D_i

1.0 Introduction

1.1 The biological importance of copper

Copper is an essential trace element in animal nutrition, all animals and plants require an intake of copper in order to grow and develop. The nutritional requirement stems from copper's reduction-oxidation (redox) properties providing an essential role in the function of numerous cuproproteins and cuproenzymes. In mammals, copper is vital for respiration and metabolism with roles in; oxidative phosphorylation, neurotransmitter synthesis and denaturation. Copper is required for around 300 different proteins, over 20 metalloenzymes and cofactors and functions in a multitude of body processes including; antioxidation, pigmentation and formation of fur; wool, connective, bone and myelin tissues; hormone release, iron metabolism and acts indirectly in haematopoiesis (Cerone *et al.*, 2000; Crisponi *et al.*, 2010; Suttle, 2010; De Sousa *et al.*, 2012).

Copper is able to convert between monovalent, cuprous (Cu^{+}), and divalent, cupric (Cu^{2+}), forms (Crisponi *et al.*, 2010). This ability of copper to easily accept and donate electrons underpins its important role in redox reactions and in scavenging reactive oxygen species (ROS): unpaired electrons which result from cellular activities and are able to damage cells through oxidative stress (Linder *et al.*, 1998).

Copper's role as an essential, biological, redox factor is strongly influenced by its properties (Boal and Rosenzweig, 2009; Crisponi *et al.*, 2010). It has high solubility (Cu^{2+}) and high polarizability. It can form linear and trigonal co-ordination with soft bases (S or N-ligands) and to a lesser extent hard bases (O-ligands), and has a great affinity for thiol groups (SH); which result in high stability constants for copper compounds making them suitable for movement and transport (Boal and Rosenzweig, 2009; Crisponi *et al.*, 2010).

Conversely, the properties which make copper essential also make copper toxic in excess; due to its ability to bind to sulphur-containing thiol groups on proteins interrupting their function (Boal and Rosenzweig, 2009), interfering with the homeostasis of other metals through displacement, and generating ROS through Fenton reactions causing direct oxidation of cellular components such as; proteins, lipids, DNA and the lysosome which releases its hydrolytic enzymes resulting in cell death (Boal and Rosenzweig, 2009).

Therefore, control of cellular copper supply is likely to be fundamental to survival. Excess copper can be detoxified through; reducing uptake, enhancing efflux and promoting sequestering to chaperones (Dameron and Harrison, 1998). Due to its essentiality, a buffer or reserve must also be maintained within each cell which can be mobilised when cellular supply is insufficient (Nevitt *et al.*, 2012). It is critical that in all living organisms intracellular copper is regulated; maintaining an equilibrium between requirement, deficiency and toxicity (Figure 1) (Vulpe and Packman, 1995; Knight *et al.*, 1996; Zhou and Gitschier, 1997).

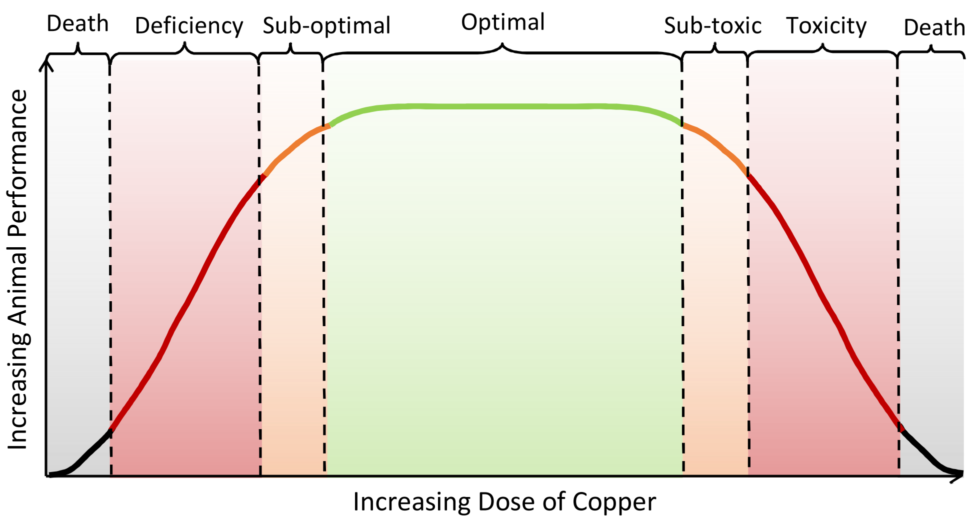

Figure 1: Dose-response curve for animal performance vs copper supply (Kendall, 2014).

1.2 Copper imbalance

Copper is considered to be imbalanced where the supply fails to provide, or exceeds, optimal (Figure 1); commonly manifesting as deficiency or toxicity. In many cases the imbalance may be marginal or sub-clinical. However, copper is considered to be the second most limiting mineral in ruminant metabolism, after phosphorous (Mackenzie *et al.*, 1997a; Arthington, 2003). In the UK, copper imbalance, as a result of both deficiency and toxicity, was the most reported mineral problem between 2004 and 2014 (AHVLA, 2012, 2014). There were ~300 fatal occurrences each year reported for cattle and sheep combined, which likely represent multiple animal fatalities per occurrence (AHVLA, 2012, 2014; Sinclair and Mackenzie, 2013).

1.2.1 Copper deficiency

Copper deficiency causes clinical signs such as anaemia, reduced growth and general 'ill thrift' (Gooneratne *et al.*, 1989a; NRC, 2000, 2001; Suttle, 2010). In adults, abnormality in osteogenesis, especially in the long bones of the limbs, can lead to fragility resulting in fractures (Gooneratne *et al.*, 1989a). Degenerative heart disease and potential heart failure can develop, sometimes termed 'falling disease' (Gooneratne *et al.*, 1989a; NRC, 2000, 2001; Suttle, 2010). Immune function can also decline increasing susceptibility to infection and commonly diarrhoea; often termed 'scours' is observed (Gooneratne *et al.*, 1989a; NRC, 2000, 2001; Suttle, 2010). One of the early and more characteristic clinical signs is de-pigmentation of the hair; achromotrichia, and a loss of hair around the eyes which gives rise to the 'spectacled' appearance of cattle (Gooneratne *et al.*, 1989a; NRC, 2000, 2001; Suttle, 2010; Peers and Phillips, 2011). Reproductive status is also affected; depressed or delayed oestrus, infertility and a higher neonatal mortality rate are commonly associated with low copper (Phillippo *et al.*, 1985, 1987b; Gooneratne *et al.*, 1989a; NRC, 2000; Spears, 2001; Suttle, 2010). Younger animals exhibit poor growth, loss of body and coat condition, and new-born animals may present with neonatal ataxia, commonly termed 'swayback' (Chalmers, 1974; Gooneratne *et al.*, 1989a; NRC, 2000, 2001; Suttle, 2010). These clinical signs are not always present in animals of low copper status, highlighting one of the many difficulties in evaluating appropriate copper supply (Suttle *et al.*, 1970; Mills *et al.*, 1976).

1.2.2 Copper toxicity

Copper toxicity can be acute or chronic. Chronic toxicity, also called chronic copper poisoning, occurs when the liver reaches its capacity for copper. This can vary with different animals, physiological stages and notably with species and breed (Menzies *et al.*, 2003). These 'copper loaded' animals can continue in this laden state until a stress on the liver as simple as; a dietary change, turning out to pasture, infection, or change in social group, triggers a haemolytic crisis

(Bidewell *et al.*, 2012; Kendall *et al.*, 2015). The pre-haemolytic phase involves the slow accumulation of hepatic copper over a period of time, ranging widely from weeks to years (Van Ryssen *et al.*, 1986; Bidewell and Livesey, 2004; Suttle, 2010). Under practical conditions, copper loading may continue without showing clinical signs (Kendall *et al.*, 2015). Although hepatic histochemical changes in the liver enzymes; sorbitol dehydrogenase, arginase, and glutamic dehydrogenase can be detected (Gooneratne *et al.*, 1980; Kaneko *et al.*, 1997). Plasma copper concentration does not rise progressively during accumulation, but surges at the onset of liver necrosis as copper floods into the blood stream (Bidewell *et al.*, 2012). This is the point of haemolytic crisis and occurs in acute copper poisoning causing severe clinical signs from which recovery is unlikely (Larson, 2005).

The onset of haemolytic crisis lasts only 2-6 days. Hepatocytes become saturated with copper causing the lysosome to rupture, resulting in swelling and necrosis of the hepatic parenchymal cells and Kupffer cells leading to focal necrosis (Gooneratne *et al.*, 1980; Kaneko *et al.*, 1997; Bidewell *et al.*, 2012). The lesions and damage that arise are thought to be copper-initiated oxidative damage from ROS (Kumaratilake, 2014). The surge of copper elevates plasma copper concentrations, which elicit intravascular haemolysis followed by; haemoglobinuria, jaundice and methaemoglobinaemia (Bidewell *et al.*, 2012). The haemolysis is considered to be a result of changes within the erythrocyte rather than a direct effect of copper on the erythrocyte membrane (Kaneko *et al.*, 1997). The copper and iron released into systemic circulation accumulate in the kidneys causing tubular nephrosis and lysosomal rupture; presenting 'gun-metal grey', haemoglobin-stained, kidneys at post-mortem (Kaneko *et al.*, 1997; Suttle, 2010). In the post-haemolytic phase, even for animals which survive, morbidity is high and characteristic kidney damage is evident (Gooneratne *et al.*, 1980).

1.3 Copper requirement

The requirement for copper differs greatly between ruminant species, breed and life-stage. In general, cattle are considered to be more tolerant to copper than sheep. Sheep are thought to have a diminished ability to excrete copper and subsequently accumulate hepatic copper (Lockhart *et al.*, 2000). Texel sheep have a high sensitivity to copper (Menzies *et al.*, 2003).

Breeds such as the North Ronaldsay are particularly sensitive to copper and have an increased susceptibility to copper-induced oxidative stress; having adapted to an environment with a low forage copper content (Haywood *et al.*, 2000; Van Metre, 2000; Simpson *et al.*, 2006). In cattle too, there are reported breed differences in copper tolerance. Holstein cows are understood to be more tolerant to elevated copper than Jersey cattle (Du *et al.*, 1996). While Simmental and Charolais cattle are thought to be more susceptible to copper deficiency than Angus cows (NRC, 2000).

The National Research Council (NRC) guidelines state copper requirements for dairy cattle should be fulfilled by 12-16 mg/kg dry matter (DM), depending on their life-stage (NRC, 2001). For beef cattle the requirement sits at 10 mg/kg DM (NRC, 2000). In sheep, requirements are much lower between 4-10 mg/kg DM (Menzies *et al.*, 2003), and goats much higher between 15-25 mg/kg DM (NRC, 2007; European Food Safety Authority, 2016).

These guideline values have been calculated using endogenous losses of copper predominantly from faeces. However, a proportion of faecal copper results from excess absorbed copper and copper which has been egested rather excreted, thus not all faecal losses need to be replaced (Suttle, 2010). Endogenous stores of copper are varied, the liver contributes the highest concentration, while most carcass components such as muscle, fat and bone are relatively low in copper, and milk also contains comparatively low concentrations. Of the endogenous stores, excluding the liver, muscle is the largest contributor containing between 0.8-1.2 mg/kg WW in cattle and sheep (Grace, 1983; NRC, 2001; Suttle, 2010). However in sheep, the fleece accounts for a relatively large endogenous store (NRC, 2007).

Studies have found that 13-14 mg Cu/kg DM was sufficient to maintain performance and reproduction in dairy heifers, while increasing copper intake to 16 mg/kg DM resulted in a rise in hepatic copper concentration (Dairy Co, 2012a; Kendall and Bone, 2013). For high yielding cattle 17 mg/kg DM was sufficient to maintain copper status, and feeding 20 mg/kg DM increased hepatic copper concentration substantially (Dairy Co, 2012a; Kendall and Bone, 2013; Sinclair *et al.*, 2017). Feeding 20 mg/kg DM also showed a negative effect on finishing cattle gain and feed efficiency (Engle and Spears, 2000). The Advisory Committee on Animal

Feedingstuffs (ACAF) guidelines recommend that 20 mg Cu/kg DM in cattle should be sufficient in the majority of circumstances and where this level is to be exceeded caution should be exercised (Bone *et al.*, 2011).

The UK maximum permitted level (MPL) of copper in bovine diets without veterinary prescription, until recently, was 40 mg/kg DM (calculated from 35 mg/kg complete feed at 88% DM) (Bone *et al.*, 2011), which has now reduced for all adult bovines to 34 mg/kg DM (calculated from 30 mg/kg) (European Food Safety Authority, 2016). In sheep, the MPL remains 15 mg/kg DM (European Food Safety Authority, 2016). From the evidence it is debated that even this newly reduced limit may be exposing cattle to chronic copper toxicity and it has been previously argued that safe dietary copper levels cannot be effectively defined (Ward, 1978).

1.4 Copper supply

1.4.1 Copper content of feedstuffs

The copper content of forage can range between 4-10 mg/kg DM (Gooneratne *et al.*, 1989a), and can be effected by seasonality; decreasing during summer and increasing during late autumn (Clarkson and Kendall, 2018). The copper content of forages appears to be a saturable process and is not predictable based on soil concentrations (Badilla-Ohlbaum *et al.*, 2001). A soil-plant barrier exists which limits copper uptake from the soil at <35 mg/kg even where soil content is increased to 4,000 mg/kg. Copper appears to reach this saturation point due to root damage caused by the increased copper concentration (McBride, 2001).

The copper content of pastures and forages varies with the species, strain and maturity of the plant, in general, grass is considered to be low in copper with brassicas and cereals providing a higher source (Suttle, 2010; Dezfoulian *et al.*, 2012). In the UK, grass copper content generally falls between 2-10 mg/kg DM, with typical values quoted ~8 mg/kg DM (AHDB, 2013; British Grassland Society, 2017). Copper is also unevenly distributed in temperate grasses, with leaves containing higher concentrations than stems and temperate grasses tending to contain less copper (4.7 mg/kg DM) than legumes (7.8 mg/ kg DM) grown in the same conditions (Suttle, 2010). A survey of forage samples across the USA reported 14% were deficient (<4 mg/kg DM), 50% were marginal (4-7 mg/kg DM), and 36% adequate (>7 mg/kg DM) in copper content (NAHMS, 1996). Another survey found corn silage contained the least copper (<5 mg Cu/kg DM), followed by hay and other forages at 6 mg/kg DM, and haylage contained the most copper at 7 mg Cu/kg DM (Vaage *et al.*, 1999). While forages tend to be lower than requirement guidelines, cereal-based concentrates are formulated to routinely contain between 10-20 mg Cu/kg DM (Suttle, 1977; European Food Safety Authority, 2016).

1.4.2 Copper content of supplemented diets

In the UK, it is almost ubiquitous for lactating dairy cattle to be supplemented with copper but much less routine in; dairy heifers, dry cows, beef cattle and sheep. An audit of UK dairy farms found mean copper intake was in excess of requirement (discussed in Section 1.3) at 28 mg/kg DM, ranging from 13 mg/kg DM; within requirement parameters, to 58 mg/kg DM which exceeds the MPL (Sinclair and Atkins, 2015). The data was further analysed to show average copper intakes were in excess of requirement during early lactation (28 mg/kg DM) and in late lactation (21 mg/kg DM) (Sinclair and Mackenzie, 2013). It was also found that when all copper input was accounted for 62% of UK dairy farms supplemented above 20 mg/kg DM, with 20% feeding over 30 mg/kg DM and 8% feeding over 40 mg/kg DM (Dairy Co, 2012b). Excess intake of copper appears in the USA too, where mean copper intake was found to be 19 mg/kg DM (Li *et al.*, 2005). In this study, 94% of dairy farms were feeding above the NRC guideline requirement for the type of dairy cattle, with some farms in the audit supplementing over 40 mg/kg DM (Li *et al.*, 2005).

Beef cattle are not exempt from oversupply. Feedlot nutritional consultants across the USA were surveyed in 2007. Their recommendations exceeded the NRC guideline for beef in many cases. In fact, this was the minimum inclusion rate advised. The maximum inclusion rate was 40 mg/kg DM, with a mean of 18 mg/kg DM and a mode of 20 mg/kg DM (Vasconcelos and Galyean, 2007). An updated survey 9 years later, found the minimum and mode inclusion rates remained the same. However, the maximum inclusion rate had increased to 50 mg/kg DM, increasing the mean to 20 mg/kg DM, despite the NRC guideline remaining at 10 mg/kg DM

(Samuelson *et al.*, 2016). Troublingly, 36% of consultants stated they did not consider the trace mineral content of the basal diet before formulating feeds (Samuelson *et al.*, 2016).

1.5 Digestion in ruminants

Ruminants are herbivorous mammals with a unique digestive system. The most remarkable aspect of their digestion is their four-chambered stomach comprising; the rumen, reticulum, omasum and abomasum (Fuller, 2004). The rumen is the foremost compartment connected to the mouth via the oesophagus. It creates a continuous compartment with the reticulum; a muscularly walled pouch with a rough, reticulated lining for physical breakdown of particulate matter (Fuller, 2004). The rumen compartment accounts for ~85% of total stomach volume and has a large capacity; 50-100 l in adult cattle and 11-19 l in adult sheep (Rémond *et al.*, 1996; Poonia *et al.*, 2011). It is responsible for the fermentative digestion of plant matter through the secretion of enzymes produced by a microbial population (Nagaraja, 2016). The inner surface of the rumen is covered with papillae which serve to increase the surface area for absorption. The papillae vary in their morphology across the rumen surface and show variation across ruminant species (Rémond *et al.*, 1996; Poonia *et al.*, 2011). The observed variation in papillae appears to be connected with the rumen cycle and the layering of the rumen contents.

The rumen contents are heterogeneous comprising a solid, liquid and gas phase. The gas located in the dorsal region is predominantly composed of carbon dioxide (~65%) and methane (~35%) although small amounts of; hydrogen, nitrogen and oxygen are also present (Nagaraja, 2016). Below the gas phase lies the solid and liquid phases. The solid phase is composed of a complex mass of digesta which floats or sediments within the liquid phase depending on its density, and can vary greatly in particle size from roughly masticated to fine particulate (Nagaraja, 2016). The most recently ingested, lighter, and lesser digested matter forms a layer on top of the rumen fluid known as the rumen mat. As fermentation takes place, the rumen contracts passing material into and out of the reticulum for physical breakdown; through the generation of rhythmic muscular contractions at 1-2 min intervals, mixing the digesta and allowing for eructation of excess gas (Hungate, 1975). Contractions move in a cyclical wave beginning in the reticulum and cranial sac followed by the dorsal, then the ventral sacs. The contraction of the reticulum directs the rumen fluid with its suspended particulate through the reticulo-omasal orifice into the next compartment (Fuller, 2004). Diets which are high in grain concentrates, as opposed to forage based diets, typically have a much smaller rumen mat and finer colloidal particulate comprising the solid phase (Ahvenjärvi, 2002).

The liquid phase is composed of large quantities of saliva (Mohamed and Chaudhry, 2008), and is highly populated with microbiota (Nagaraja, 2016). The saliva naturally buffers pH through sodium bicarbonate (100-140 mM) and phosphate (10-50 mM) at pH 8-8.4 which diminishes the effects of the volatile fatty acids (VFA) and lactic acid produced from bacterial fermentation (Hungate, 1975; Moran, 2005). The pH ranges in a healthy rumen between pH 5.8-6.4 (Koritz, 1982; Rémond *et al.*, 1996; Kendall *et al.*, 2001; Kamra, 2005; Moran, 2005). In grazing ruminants and those fed predominantly forage based diets, the rumen maintains a pH towards the upper end of the range. Those which consume a grain concentrate diet are towards the lower end. Below pH 6.0 ruminal microflora and VFA composition undergo changes which, as pH decreases, can lead to impairment of rumen function and inhibition of bacterial species and will finally lead to rumen acidosis; a potentially life-threatening metabolic condition, if the rumen reaches less than pH 5.5 (Zamarreño *et al.*, 2003; Zhao *et al.*, 2018).

The rumen is a dynamic environment but it also confers a level of stability which has given rise to the optimal growth for rumen bacteria at a temperature of 39°C, with a small range of 38-42°C (Bryant, 1959; Koritz, 1982; Kamra, 2005; Moran, 2005). These conditions within the rumen are maintained by the gases generated during bacterial fermentation. In order to thrive and grow the bacteria which inhabit the rumen require; carbon dioxide, nitrogen, sodium, and VFAs (Hungate, 1975). The rumen creates a reducing environment. Reducing environments are defined as having a redox potential below 100 mV and are known to inhibit natural oxidation. The rumen has a low redox potential around -350 mV allowing only microbiota which are able to tolerate this low redox potential to survive (Kamra, 2005; Nagaraja, 2016). The environment within the rumen is predominantly anoxic (without atmospheric di-oxygen) and hosts both obligate and facultative anaerobes (Holovská *et al.*, 2002). Small quantities of oxygen may be present during ingestion and rumination creating an oxygenated region close to the rumen wall (Richter, 2011). Most of the ruminal obligate anaerobes are known to be highly sensitive to oxygen and exposure is lethal to these species. However, there is some variance in their ability to tolerate oxygen between <0.5 to 8% which is considered to reflect the small quantity of superoxide dismutase and catalase; cellular enzymes for the detoxification of ROS, they are able to produce (McCord *et al.*, 1971; Holovská *et al.*, 2002). Despite their tiny amounts of antioxidant enzyme production, it has been demonstrated that there is a measurable amount of activity; sufficient to consume and detoxify small amounts of oxygen that may be found in the rumen (Fulghum and Worthington, 1984). The oxygen created through gas exchange at the rumen wall and swallowed alongside feed is used by facultative anaerobic bacteria preserving anoxic conditions suitable for the obligate anaerobic bacteria (Kamra, 2005). The rumen microbiota comprise; bacteria (40-90% total microbial mass), protozoa (0-60%), fungi (~10%), methanogens (2-4%) and a negligible proportion of bacteriophage (Nagaraja, 2016). The population density of the microbiota is considerable and ranges from 10^8 to 10^{12} per g of rumen contents (Nagaraja, 2016). Although, this can be altered by diet, with grain-based populations ranging from 10-100 times more than the forage based diets (Nagaraja, 2016). The bacterial population are mostly associated with feed particles (~70%) and free-floating bacteria within the liquid phase make up the lesser component (~30%) (Nagaraja, 2016).

The rate of digestive transit and retention of digesta can vary with diet and there have been reported differences with breed (Voigt *et al.*, 2000), as well as those expected with species due to rumen size and total tract length (Grovum and Williams, 1973). Total digestive processing times in cattle are between 40-60 h; with processing in both the rumen and reticulum comprising the majority of this time (Peyraud *et al.*, 1989; Mambrini *et al.*, 1994). Reticulorumen retention times in cattle have been averaged for concentrates at 23 h, forages at 29 h and silages at 40 h, although retention could be as long as 60 h. Text books indicate the average falls between 20-30 h (Koritz, 1982; Peyraud *et al.*, 1989; Mambrini *et al.*, 1994; Beltran *et al.*, 2015). In sheep, the retention time is much shorter, between 10-14 h consistent with a much smaller rumen capacity (Grovum and Williams, 1973).

After ruminal processing the transit is much faster. There is a flow of fine particulate and rumen fluid into the omasum with each contraction. The omasum has a unique lining comprising deep tissue folds that create an absorptive area equivalent to several m^2, although the compartment is small, containing less than 8 l in cattle (Fuller, 2004). The omasum is responsible for the reabsorption of saliva and acts as a filter preventing undigested particulate from leaving the rumen. The final stomach compartment follows the omasum. The abomasum is the gastric or 'true' stomach and shares a function and morphology with the monogastric stomach. It is the site of enzymatic protein breakdown and is maintained at a constant pH of 2.1-2.2 created by the biological secretion of hydrochloric acid at pH 1.0-1.3 (Koritz, 1982; Cao *et al.*, 2000; Moran, 2005). The retention time in the abomasum is very short in comparison to the rumen; between 30-45 mins. Although in some cases it may be retained for up to an hour in cattle (Mambrini *et al.*, 1994; Ahvenjärvi, 2002) and as short as 15-40 mins in sheep (Grovum and Williams, 1973).

After processing in the stomach, the ruminant digestion progresses in a similar manner to monogastric digestion with a small intestine comprising duodenum; responsible for enzyme secretion and maintaining a higher pH ~8, followed by an ileum and jejunum; lined with villi and microvilli for nutrient absorption, a functional caecum and finally a large intestine complete the tract (Fuller, 2004).

1.5.1 Copper absorption in ruminants

Copper is primarily absorbed in ruminants in two main regions; across the stomach, in particular the abomasum, and small intestine. Although, some absorption of copper may also occur in the colon which is of more relevance to sheep than cattle (Ivan and Grieve, 1976; Turner *et al.*, 1987; Gooneratne *et al.*, 1989a; Hussein, 2017). There is also some evidence that copper is able to be absorbed in a minimal capacity across the rumen epithelium (Linder and Hazegh-Azam, 1996; Wapnir, 1998; Wright *et al.*, 2008; Safdar and Kor, 2014). For copper to be absorbed across the apical membrane it must be in its reduced (Cu^+) form. The high affinity copper transport protein present in the enterocytes is able to transport Cu^+ from the digestive fluid overlying the apical membrane of the gastric and intestinal brush border and is able to absorb as much as 40-60% of the soluble copper across the epithelium (Goff, 2017). A full review of copper trafficking in the enterocytes is described in Clarkson *et al.*, (2019).

1.6 The copper conundrum

Monogastric animals which possess a simple gastric stomach have a direct correlation between copper supply and physiological copper status (Suttle, 2010). Death from copper deficiency and toxicity is rare in monogastric animals (Suttle, 2010). Copper requirements for these species are much lower and less varied; ~6 mg/kg DM in dogs, pigs and rats (European Food Safety Authority, 2016). However, copper provision is not straightforward in ruminants; creating the copper conundrum.

Copper deficiency is caused through low dietary supply of copper or the use of copper sources which remain insoluble during digestion; and are thus unavailable for absorption. Copper deficiency from these causes is termed a 'primary deficiency' (Ward *et al.*, 1996; Olson *et al.*, 1999). Under practical conditions, an insufficient supply of copper leading to primary copper deficiency is likely to be rare in the UK (Bone, 2010). However, unique to ruminants, a perceived deficiency can occur where copper intake is adequate but interactions with copper antagonists: dietary elements which are able to interact or bind with copper and subsequently inhibit its uptake or bioactivity (Suttle, 1991; Olson *et al.*, 1999; Spears, 2003; Gould and Kendall, 2011). Perceived deficiency from this cause is termed 'secondary deficiency'.

The interactions with other dietary elements act through several mechanisms. They can form insoluble complexes, or complexes too large for absorption, or, introduce competition for absorption transporters in the small intestine (Gould and Kendall, 2011; Spears, 2013). Since there is minimal absorption of copper across the rumen epithelium, complexation in this region results in unavailable copper in the intestine; its principal region of absorption, and is subsequently lost through the faeces (Safdar and Kor, 2014). The problem is further complicated where antagonists, without a copper component are absorbed and are able to interfere with the function of systemic copper (Gould and Kendall, 2011). Discussed in more detail in Sections 1.7-1.11.

The unique anatomy of the ruminant stomach, with its anaerobic fermentation and ligand rich environment (discussed in Section 1.5) is recognised as the catalyst for secondary deficiency to occur and produces a marked effect on copper absorbability (Suttle, 2012). Absorbency values from dietary copper in adult ruminants range between 1-10% in comparison to ~70% in monogastric species (NRC, 2001; Spears, 2003). Prior to developing full rumen function, copper absorption is around 70-85%. However, this absorption level drops at weaning after the closure of the rumino-reticular groove; which allows the milk to by-pass the rumen (Spears, 2003). The theory that a functional rumen inhibits copper absorption was demonstrated through the introduction of rumen ciliate protozoa to fauna-free sheep. Sheep without rumen fauna succumbed to copper toxicity and exhibited a higher plasma copper concentration, due to increased absorption from an un-adjusted dietary ration; despite having a lower dry matter intake (DMI) (Ivan *et al.*, 1986; Ivan, 1988).

It has been speculated that in the absence of interactions with other elements, the absolute dietary requirement for copper could be considerably lower than the current requirement; since endogenous losses of copper are only ~7 µg/kg body weight (BW) (NRC, 2001). It was estimated that the requirement for metabolically available copper, calculated based on body weight and average daily gain in steers, could be ~1.96 mg/day (Wittenberg *et al.*, 1990b), as such, it is suggested that the requirement for copper could be lower than 1 mg/kg DM (Zervas, 1983; Moeini, 1997; Bone, 2010, N. R Kendall 2019, personal communication, 27 May).

1.6.1 Practical impact of the copper conundrum

The ruminant livestock industry is aware a problem with copper deficiency exists and that due to antagonist presence, copper provision is not straightforward. However, evidence gathered from industry professionals indicates it is inconsistently understood among this demographic (Black and Kendall, 2010). Vets vary in their response to copper-related problems, some may discourage supplementation in fear of toxicity problems, while others may continue to recommend supplementation regardless of current practices and status (Black and Kendall, 2010; Bidewell *et al.*, 2012; Bowyer, 2016; Hunt, 2016). As discussed in Section 1.4.2, consultants may promote supplementation without assessing need (Vasconcelos and Galyean, 2007; Samuelson *et al.*, 2016), leading to supplementation with very little evidence of deficiency risk (Livesey *et al.*, 2002; Cutler and Jones, 2003; Laven and Livesey, 2004; García-Vaquero *et al.*, 2011; López-Alonso *et al.*, 2017). Historically, the industry has considered cattle to be tolerant of relatively high copper, especially in comparison to sheep. Concern for copper deficiency has led to the inclusion of a 'safety margin' in most cattle diets (Sinclair and Mackenzie, 2013). It seems that both vets and farmers have been persuaded that the 'worst-case scenario' prevails (Suttle, 2016). Advice suggests inclusion under normal conditions, without antagonist presence, of 120-150% of NRC guidelines (suggested 17-20 mg/kg DM) will provide effective risk management without accumulation (Weiss, 2005, 2017), and where diets are entirely pasture based as much as 200% of the NRC guideline may be recommended (Weiss, 2005, 2017). Interestingly, this advice stresses the potential for accumulation and toxicity, and mentions that exact inclusions are not able to be reliably calculated, but fails to take into account whether there is any presence, or effect, of antagonist elements.

There is also misunderstanding of the terminology used in expressing copper requirement. A vet or nutritionist may consider 'adequate' to be synonymous with 'sufficient' (Grace and Knowles, 2015). However, 'adequate' appears to imply 'could be better' to non-professionals; who subsequently view upper values as targets instead of limits, promoting unnecessary supplementation (Grace and Knowles, 2015).

There is considerable marketing pressure from industry press and supplement manufacturers suggesting copper supplementation is necessary (Bidewell *et al.*, 2012; Bowyer, 2016; Hunt, 2016), and moreover, choice of supplement could be key to avoiding problems (Bone, 2011; Cheng *et al.*, 2011; Animax, 2017; ADM, 2018; Meads, 2018; Kleinschmit, 2019). Farmers face marketing pressure to 'fix' problems such as underperformance in fertility with trace element supplementation. Such fixes appeal to farmers as it suggests their problem lies beyond management strategy and therefore, they are not at 'fault' (Whitaker, 1999).

From the producer's viewpoint clinical signs impact on animal health and performance. Farmers may supplement attempting to avoid deficiency but it fails to alleviate clinical signs. Thus, farmers increase supplemental copper; potentially exposing their herd or flock to copper accumulation. This supplemental pattern is also observed where a little supplementation gives a small improvement. Leading to the temptation to provide more, thinking it will give a greater improvement (Kendall, 2014).

When deciding to supplement, some sources of copper intake may be overlooked, such as; free-access minerals, boluses, forages, water supply or ingested soil; from dry conditions or low sward pasture, all of which can contribute to total copper intake (Healy, 1974; Sinclair and Mackenzie, 2013; Kendall and Bone, 2019). Regular analysis or calculation of complete copper intake is rare as part of routine ration formulation (Dairy Co, 2012b; Kendall and Bone, 2019). As is the analysis of the copper antagonists, or adjustment of copper input in line with their presence; leading to a poor correlation between copper supply and risk of deficiency (Dairy Co, 2012b). Among those who do analyse forage, the resulting mineral report is a useful indicator of absolute mineral content, but the 'copper availability index' can be misleading; and promotes unnecessary copper supplementation (Bone, 2010).

In addition, problems in gaining an accurate assessment of copper status in both herds and individuals is an important factor creating difficulty in making an informed decision (Sinclair and Mackenzie, 2013). Combined, all these factors lead to a culture of supplementation; which Black and French (2000) observed farmers could not be persuaded to cease if they believed it to be essential.

Indicators from academic studies, government reports and industry, suggest the copper status of UK ruminants may be getting further from optimal rather than closer (Bidewell *et al.*, 2012; Alderton, 2015; Kendall *et al.*, 2015; European Food Safety Authority, 2016; Hunt, 2016; Price, 2016, 2017; AHPA, 2019; Strickland *et al.*, 2019a). The abundance of animals with high copper intakes and elevated copper status raises the question of why anecdotal sources report that copper deficiency is still perceived to be common (Glanvil, 2017; Gowling and Hardy, 2019; Swire, 2019a, 2019b). Contributing to this perception is the UK farming press who routinely publish content pertaining to the need for routine copper supplementation; arguably above any other trace mineral concern (Laven and Livesey, 2004; Glanvil, 2017; Priestley, 2018; Gowling and Hardy, 2019; Swire, 2019a, 2019b).

1.7 Trace element interactions: Molybdenum and Sulphur

The existence of an interaction between sulphur, molybdenum and copper has been observed since the 1950s (Dick, 1953). In 1975, this was attributed to the formation of thiomolybdate; an anionic sulphur-molybdenum complex derived from molybdate, where one or more of its oxygen atoms are replaced by sulphur (Dick *et al.*, 1975).

It was initially established that feeding molybdenum alongside sulphur to ruminants resulted in decreased copper absorption, which in turn, negatively impacted markers for biological copper status such as; plasma copper, caeruloplasmin and hepatic copper (Dick, 1953; Mills, 1960; Smith *et al.*, 1968; Suttle and Field, 1969; Marcilese *et al.*, 1970). The ability of molybdenum to effect copper absorption is inherently linked to the availability of ruminal sulphide (Mills, 1960; Dick *et al.*, 1975; Spears, 2003). The importance of sulphur in this interaction was demonstrated through the inclusion of increasing amounts of molybdenum (from 0.5 to 4.5 mg/kg) without any effect on copper bioavailability when dietary sulphur content remained low (1 g/kg) (Suttle, 1975). However, once the sulphur content was increased to 3 g/kg, the availability of copper reduced by 40-70% (Suttle, 1975).

This interaction has been deemed so significant in ruminants that it has been considered to account for the marked drop in copper absorption that occurs alongside the development of the functional rumen (Spears, 2003). As a result, the recommended nutritional requirements in ruminants are based around the use of a coefficient model developed by Suttle and McLauchlin, (1976): $\log(absorbable\ copper) = -1.153\ - 0.076\ (S\ g/kg) -\ 0.013\ (S\ \times\ Mo\ mg/kg)$. This coefficient aims to calculate the expected copper absorption based on the anticipated effects of the interaction. In reality, it is more difficult to predict the availability of copper than the equation would suggest.

It has been noted that the effect of the molybdenum-sulphur interaction can vary, not only with copper source, but also with feedstuff. Suttle, (2010) reported that absorbable copper in fresh grasses was the most affected by the molybdenum-sulphur interaction. Grasses appeared to have a lower absorbable copper than hay and ensiled grass, and any addition of sulphur or molybdenum at any concentration drastically reduced copper absorption. In comparison, grass silage was less affected by an increase in molybdenum, but was greater affected by increased sulphur in the ration. Whereas in hays, the effect of molybdenum was relatively small, but an increase in sulphur content from 0.2-0.4% decreased the absorbable copper markedly (Suttle, 2010).

Thiomolybdate can exist in a range of species from mono to tetra-thiomolybdate. As the oxygen atoms in the complex are replaced by sulphur atoms, the affinity for copper increases; making tetra-thiomolybdate the most potent of the species (Quagraine, 2002; Gould and Kendall, 2011). All species of thiomolybdate have been demonstrated to form under rumen replicated conditions. Early *in vitro* work showed around a third of molybdate could be converted into tri and tetra-thiomolybdate where the S:Mo ratio was less than 10:1 (Clarke and Laurie, 1980; Bray *et al.*, 1982a; Mason, 1986b). More recent *in vitro* work found that within 5 minutes all molybdate present had reacted forming a sequential pattern of species, shown in Figure 2 (Quagraine, 2002).

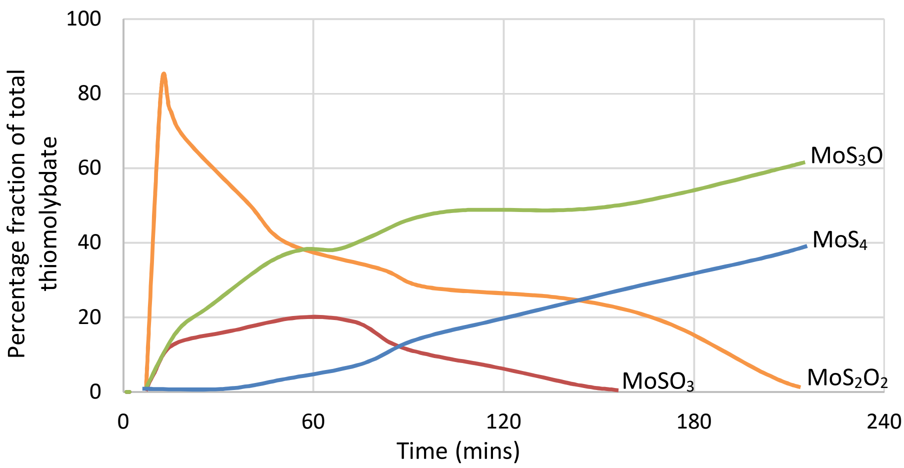

Figure 2: Percentage formation of thiomolybdate species; mono-thiomolybdate ($MoSO_3$), di-thiomolybdate (MoS_2O_2), tri-thiomolybdate (MoS_3O) and tetra-thiomolybdate (MoS_4) after mixing sodium molybdate ($NaMoO_4$) with ammonium sulphide at a S:Mo ratio of 22:1 at pH 7, 38 ˚C and an ionic strength of 0.2 M to replicate rumen conditions. Data as reported in Quagraine, (2002).

Although, this provides evidence that tri and tetra-thiomolybdate are likely to be the most prevalent species in the rumen. The *in vitro* models cannot fully replicate the intricacy of the rumen environment; resulting in molybdenum associating with the liquid phase instead of the solid phase (Mason, 1986b). Therefore, under practical rumen conditions the proportion and species formed may vary to these models. Tetra-thiomolybdate has been noted to form where rumen pH is slightly more acidic while tri-thiomolybdate is the most prominent species at pH 6.2 (Gould and Kendall, 2011).

The antagonism proceeds as a two-step process starting in the rumen; when dietary concentrations of molybdenum and sulphur are sufficiently high (Spears, 2003). First, sulphur combines with molybdenum to form thiomolybdate. Secondly, thiomolybdate binds with copper forming an insoluble copper-sulphur-molybdenum compound (Price *et al.*, 1987; Gooneratne *et al.*, 1989a; Suttle, 1991; Galbraith *et al.*, 1997; Spears, 2003). Through its sequestering potential, thiomolybdate acts to significantly reduce the exchangeable copper in the rumen and ultimately reduces copper available for absorption (Chidambaram *et al.*, 1984; Essilfie-Dughan, 2007). However, in the absence of an adequate amount of available rumen copper thiomolybdate is able to absorb into the animal through the wall of the rumen or small intestine (Kelleher *et al.*, 1983; Price *et al.*, 1987; Mackenzie *et al.*, 1997b; Quagraine, 2002). Once in the systemic circulation, thiomolybdates exert their high affinity for copper co-ordinated by thiol groups, in preference over any other functional group (Ogra *et al.*, 1999). They circulate in the blood plasma in an albumin-bound complex (Gould and Kendall, 2011) and are capable of interfering with copper metabolism through binding to the copper component of biological compounds, including; caeruloplasmin, superoxide dismutase (SOD) and metallothionein (Gooneratne *et al.*, 1989a; Kumaratilake and Howell, 1989; Ogra *et al.*, 1999; Laven and Livesey, 2005). Thus, inhibiting the function of copper for biochemical processes (Spears, 2003). Sequestering by thiomolybdate does not remove the copper component from the biological compound, but renders it unable to perform in redox reactions through the formation of a stable complex (discussed in more detail in Section 6.1.5) (Hynes *et al.*, 1985; Van Ryssen *et al.*, 1986; Spears, 2003; Gould and Kendall, 2011). Thiomolybdate has a higher affinity for copper than any other essential metal. It has no effect on the metabolism of other metals with similar properties such as; iron, zinc or cadmium, even where they appear in the same chaperone (Ogra *et al.*, 1995, 1996).

Historically, this interaction was considered to be characteristically limited to 'teart' pastures; classified by a molybdenum content >20 mg/kg DM (Muir, 1941; Laven, 2018). Since the mean UK herbage concentration is reported to be as low as 0.8 mg Mo/kg DM (Livesey *et al.*, 2010), copper problems caused by dietary molybdenum should be relatively rare. However, there is evidence to suggest that the problem is widespread (Bone, 2010), and pastures as low as 0.5 mg/kg DM have identified molybdenum induced copper problems where sulphur content is sufficiently high to elicit them (Bone, 2010; Axelson *et al.*, 2018; Laven, 2018).

Copper absorption appears most inhibited when sulphur content is ≥3 g/kg and molybdenum content between 4-6 mg/kg (Suttle, 1983; Gengelbach *et al.*, 1994). Beyond this inclusion, synthesis of thiomolybdate may plateau; possibly linked to an inhibiting effect of increasing molybdenum on sulphide production (Mills, 1960; Suttle, 1983; Gengelbach *et al.*, 1994; Spears, 2003). Testing of duodenal digesta from molybdenum treated grass revealed a relative copper bioavailability eight times less than untreated grass, demonstrating the potential potency of this pathway (Wapnir, 1998).

1.7.1 Molybdenum toxicity

Molybdenum is also an essential element. It has roles in enzymes such as; xanthine oxidase, aldehyde oxidase and sulphite oxidase (NRC, 2001; Anke *et al.*, 2010). The requirement for molybdenum is so low that deficiencies have not been reported under practical conditions and its requirement has not been established, but is presumed <100 µg/kg DM (Pott *et al.*, 1999; NRC, 2001; Anke *et al.*, 2010). Like most essential elements, molybdenum can also be toxic in excess; a condition which is termed molybdenosis (Mason, 1986b; Anke *et al.*, 2010).

Molybdenum in its hexavalent state (Mo^{6+}) is readily absorbed (75-97%) in both ruminants and monogastric species from the duodenum and jejunum in water soluble forms such as; molybdate; the form most commonly found in feedstuffs, thiomolybdate, and oxo-thiomolybdate (European Commission, 2003; Anke *et al.*, 2010). It appears rapidly in the blood as molybdate bound to α-2-macroglobulin, or bound to the erythrocyte membrane (Barceloux and Barceloux, 1999; European Commission, 2003) and is found deposited in kidney, liver and bone (Barceloux and Barceloux, 1999; European Commission, 2003).

Tolerance to elevated molybdenum varies with species. Monogastric animals appear most tolerant. In ruminants, cattle appear to be the most sensitive to elevated molybdenum, followed by sheep (Ward, 1978; Anke *et al.*, 2010). Mild cases of molybdenosis may only be identified through an increase in uric acid production; as a result of molybdenum's role in xanthine oxidase (Vyskocil and Viau, 1999; Anke *et al.*, 2010).

Due to the potent effect of thiomolybdate on biological copper function, it is difficult to define the effects of molybdenosis in ruminants. In addition, the term molybdenosis is commonly misused; interchangeably with, or to describe, a thiomolybdate toxicity (Anke *et al.*, 2010; Laven, 2018). However, there are subtle differences between these two conditions. Thus making it beneficial to use data from monogastric animals to try and clarify the effects of molybdenum toxicity.

Studies in monogastric species have observed the most common clinical signs of molybdenosis to be; retarded growth, skeletal abnormality, appetite suppression, liver molybdenum accumulation and diarrhoea (Comar *et al.*, 1949; Arrington and Davis, 1953; Gray and Daniel, 1954; Van Reen, 1954; Miller *et al.*, 1956). In longer term studies, suppression of fertility and reproductive function are also observed (Suttle, 1980; Vyskocil and Viau, 1999; European Commission, 2003). An increase in alkaline phosphatase (ALP) activity has been detected alongside high molybdenum intake (Van Reen, 1954). Interestingly, ALP is found in high concentrations in bone and liver, and is also present in the intestine, placenta and germ cells (Barceloux and Barceloux, 1999; European Commission, 2003). Its increased activity at the main sites where abnormality is found in molybdenosis suggest that an interruption of the function of ALP by molybdenum may be responsible for the observed clinical signs (Feaster and Davis, 1959).

In ruminants, intake of elevated molybdenum is quoted to result in clinical signs such as; severe diarrhoea, loss of coat condition, anorexia, achromotrichia, skeletal deformity, depressed fertility, liver and kidney abnormality and death (Ward, 1978; Vyskocil and Viau, 1999; Anke *et al.*, 2010). These clinical signs appear indistinguishable from those of copper deficiency, discussed in Section 1.2.1. This similarity is no coincidence. The work of Phillippo *et al.*, (1987) and Phillippo, Humphries and Garthwaite, (1987) demonstrates that copper status could be reduced in equal measure through elevated iron and molybdenum on low copper, high sulphur, diets. However, the absence of clinical signs in the iron treatment group, and their presence in the molybdenum treatment group suggests that these typical clinical signs may represent a thiomolybdate toxicity rather than copper deficiency (Suttle *et al.*, 1970; Mills *et al.*, 1976; Telfer *et al.*, 2004). Interestingly, under practical conditions, animals which have sufficient copper status are also able to express the clinical signs of thiomolybdate toxicity (Bone, 2010; Laven, 2018).

The likelihood of thiomolybdate formation in situations where elevated molybdenum occurs indicates that in most cases of ruminant molybdenosis a thiomolybdate toxicity is also occurring. While subsequently difficult to determine, the true signs of molybdenosis in ruminants may be limited to; scouring, occurring within 24 h of initial input, especially in cattle; skeletal abnormality, weight loss, depigmentation and reproductive impairment (Thomas and Moss, 1951; Erdman *et al.*, 1978; Lesperance *et al.*, 1985; Dickson, 2016). It has been suggested that scouring is linked to the effect of elevated molybdenum on sulphur oxidase in the intestine which inhibits the conversion of sulphate into sulphide allowing the excess sulphate to irritate the mucosal lining (Gooneratne, 2006).

1.8 Trace element interactions: Iron

Iron is another essential trace mineral in ruminants. The requirement for iron is low (18-50 mg/kg DM) in comparison to most forage concentrations and it is recommended to not exceed 1,000 mg/kg DM (NRC, 2000, 2001, 2007). Ruminants frequently consume high iron from; forages, especially autumn herbage, water; principally non-potable sources, and from soil; mainly from impacted or acidic soils (Coup and Campbell, 1964; Humphries *et al.*, 1983; Gooneratne *et al.*, 1989a; Spears, 2003). All of which can have a marked inhibitory effect on copper uptake and utilisation (Campbell *et al.*, 1974).

Intake of 250–1,200 mg Fe/kg can have a significant reduction on copper status in ruminants (Bremner *et al.*, 1987; Prabowo *et al.*, 1988; Mullis *et al.*, 2003). With 500-800 mg Fe/kg demonstrating a similar effect to supplementation of 5 mg Mo/kg at reducing plasma and liver copper concentrations (Humphries *et al.*, 1983; Phillippo *et al.*, 1987a). Reduced liver copper concentrations have been found in both cattle and sheep fed diets containing 150-3,200 mg/kg iron, and 250 mg Fe/kg effectively reduced liver copper in calves by 88% over 8 weeks (Campbell *et al.*, 1974; Humphries *et al.*, 1983). Elevated iron intake has also been suspected as a trigger for the onset of neonatal ataxia in lambs where dietary copper concentrations were already low (<8 mg/kg DM) (De Sousa *et al.*, 2012). It has been quoted that any dietary intake of iron >150 mg/kg DM will have an adverse effect on copper availability (Bone, 2010).

Iron has the potential to inhibit copper absorption in two different ways. The first effect is absent in monogastric animals and pre-ruminal calves, which indicates this interaction is dependent on a functional rumen (Bremner *et al.*, 1987). This effect of iron, like molybdenum, also appears linked to presence of sulphur in the rumen (Gould and Kendall, 2011). This was evidenced by an experimental diet containing 1 g/kg iron alongside low sulphur, which failed to produce deleterious effects on sheep serum or liver copper concentration (Rosa *et al.*, 1986), and a greater depletion of blood copper concentration where iron sulphate was fed in comparison non-sulphur iron sources (Standish and Ammerman, 1971). However, unlike molybdenum, the mode of iron interaction has not yet been successfully elucidated.

It is theorised that within the rumen iron could react with sulphide forming iron-sulphide complexes. These complexes could then dissociate and exchange with copper in the lower pH of the abomasum forming insoluble copper sulphide; thus reducing available copper (Suttle *et al.*, 1984; Allen and Gawthorne, 1987; Gould and Kendall, 2011). An alternative, more recent theory, is that iron sulphide could react with copper directly, forming a copper-iron-sulphide complex which is non-absorbable (Gould and Kendall, 2011; De Sousa *et al.*, 2012).

The second effect is demonstrated through a number of cell line and monogastric species studies where high dietary iron without sulphur acts as a copper antagonist (Tennant *et al.*, 2002; Arredondo *et al.*, 2006; Garrick *et al.*, 2006; Jiang *et al.*, 2013). Experimentally, the removal of supplemental sulphur failed to reduce the severity of an iron-induced copper deficiency in cattle (Gooneratne *et al.*, 1989a). This effect has been attributed to the link between copper and iron transport in the intestine creating a second interaction independent from the rumen and presence of sulphur.

Iron absorption is controlled by two main proteins, divalent metal transporter 1 (DMT1) and ferroportin (Tennant *et al.*, 2002; Linder *et al.*, 2003; Arita *et al.*, 2010). DMT1 primarily transports reduced, divalent iron (Fe^{2+}) but, has been suggested to be a physiologically relevant copper transporter in intestinal cells (Han and Wessling-Resnick, 2002; Kim *et al.*, 2008; Prohaska, 2008), despite copper also having its own transporters and uptake mechanisms; reviewed in Clarkson *et al.*, (2019). Intestinal cell line studies have demonstrated that DMT1 actively participates in copper transport. These studies found the depletion of cellular copper increased the uptake of both copper and iron, and an increase in iron decreased both iron and copper uptake (Linder *et al.*, 2003; Arredondo *et al.*, 2004, 2006). Notably, significant copper transport by DMT1 was observed for Cu^{+} under conditions of iron deficiency (Tennant *et al.*, 2002; Arredondo *et al.*, 2003). The intestinal link between copper and iron for this transporter is possibly due to the similarity of ionic radii and electron configuration of monovalent copper and divalent iron (Bremner and Beattie, 1995; Arredondo *et al.*, 2003; Jiang *et al.*, 2013). Suggesting this mechanism may be, at least in part, responsible for the observed interactions between these elements (Hansen and Spears, 2009; Fry *et al.*, 2013; Spears, 2013).

In addition to its own interactions, excess iron may also exacerbate a thiomolybdate problem. If available copper becomes bound to either sulphur or iron, it will be unavailable to bind to thiomolybdate. Subsequently, thiomolybdate will be absorbed into the bloodstream and bind to systemic copper (Gould and Kendall, 2011).

1.9 Trace element interactions: Sulphur

In addition to its role in both iron and molybdenum antagonisms, an independent interaction exists between copper and sulphur in ruminants. Sulphur at dietary levels between 2-5 g/kg DM, routinely found in ruminant diets, can have an antagonistic effect on copper (NRC, 2000, 2001, 2007). Increasing dietary sulphur in either inorganic or organic forms from 1-4 g/kg can reduce copper bioavailability in sheep fed a low-molybdenum diet by 30–56% (Suttle, 1974a; Spears, 2013). The current increase in the use of by-products like dried distiller’s grains and corn gluten feeds increases the concentration of sulphur in feeds in modern production systems beyond requirement of ~2 g/kg DM, potentially increasing the likelihood of this interaction under practical conditions (NRC, 2000, 2001, 2007; Spears, 2013).

High dietary sulphur has been shown to reduce copper absorption through the formation of insoluble copper sulphide in the rumen, which remains insoluble under the acidic conditions of the abomasum and is subsequently excreted in the faeces; without releasing the copper in a metabolisable form to the animal (Mills, 1960; Bird, 1970; Suttle, 1974a; Dick *et al.*, 1975; Ivan, 1988; Grace *et al.*, 1997; Spears, 2003).

Within the rumen, sulphur rapidly degrades into sulphide through the action of sulphur-reducing bacteria, which are able to act on both organic and inorganic sulphur sources including; sulphate, thiosulphate, sulphite, cystine and cysteine (Bird, 1970; Bird and Moir, 1971). The concentration of sulphide in the rumen has a high turnover; excess absorbs through the rumen wall, creating a gradient for continued sulphide production while any remaining sulphate can be later absorbed in the small intestine (Anderson, 1956; Bird and Moir, 1971). Sulphur-reducing bacteria are reported to be able to produce up to 97 mg/l (~3 mM) sulphide per day when 2.25 g/l (70 mM) sulphate was fed (Anderson, 1956; Bailey and Balch, 1961).

The interaction exerted on copper by sulphur is likely to be restricted to the digestive tract. Sheep with elevated copper status fed sulphur in excess did not experience any systemic reduction in copper status, but did have a reduction in absorption (Van Ryssen *et al.*, 1986), and an increase of sulphur in both high and low copper diets was able to decrease the solubility of copper in the small intestine without affecting liver copper concentration (Grace *et al.*, 1997). This was supported in grazing sheep where fertilizing pasture to increase sulphur intake to 4 g/day also decreased duodenal soluble copper by around half (Bird, 1970; Grace *et al.*, 1998). However, further increases in sulphur had no further effect on the flow of soluble copper, indicating a plateau (Bird, 1970; Spears, 2003).

1.9.1 The effect of hydrogen sulphide

Within the rumen, the sulphur reducing bacteria predominantly utilise the dissimilatory pathway of sulphur reduction (Coleman, 1960; Forsberg, 1979). The dissimilatory pathway is considered among the earliest energy-yielding processes to sustain life and is common among archaea and obligately anaerobic bacteria, notably those which evolved before the presence of atmospheric oxygen and including the *Desulfovibrio* species of the rumen (Peck, 1961; Grein *et al.*, 2013). The dissimilatory pathway is a form of anaerobic respiration that uses sulphate as a terminal electron acceptor and produces hydrogen sulphide as an end product (Muyzer and Stams, 2008; Bradley *et al.*, 2011; Grein *et al.*, 2013). It is thought that excess dietary sulphur, commonly as sulphate, upon its reduction into hydrogen sulphide within the rumen may be the major contributor to the detrimental effects of high dietary sulphur on cattle performance and health, and on the development of neurological conditions such as polioencephalomalacia (Gould *et al.*, 1997; Gould, 1998). It is thought that the excess hydrogen sulphide gas is eructated and subsequently inhaled, by-passing hepatic detoxification, and entering the circulation where it increases blood hydrogen sulphide concentration (Gould *et al.*, 1997; Kung *et al.*, 2000). It has been estimated that 70 to 80% of hydrogen sulphide gas eructated from the rumen is subsequently inhaled (Doherty and Cook, 1962). Once in systemic circulation, the hydrogen sulphide is transported to the brain where it disrupts energy metabolism, through its action as a potent cellular toxin affecting cell respiration and acts as a pro-oxidant (Gould, 1998).

Lower rumen pHs, such as those found in grain-fed ruminants, is believed to promote production of hydrogen sulphide in the rumen gas cap (Drewnoski *et al.*, 2014). It has been theorised that the lower pH increases the concentration of hydrogen ions available for hydrogen sulphide production (Drewnoski *et al.*, 2014). In feed trials using steers ruminal pH was found to be negatively correlated with rumen hydrogen sulphide concentration, until the rumen pH exceeded 5.8 (Morrow *et al.*, 2013; Morine *et al.*, 2014).

1.10 Trace element interactions: Zinc

Zinc has been reported to have an impact on copper availability in ruminants. The antagonism between copper and zinc may be due to competitive biological interactions; as the metals share similar chemical and physical properties (Bremner and Beattie, 1995). The interaction is non-mutualistic; as increased copper has not been shown to have reliable effects on zinc absorption (Bremner and Beattie, 1995).

High concentrations of dietary zinc have been reported to induce a copper deficiency; reducing liver and plasma copper concentration through inhibiting absorption and subsequent hepatic accumulation (Bremner, 1979; Bremner and Beattie, 1995; Blanco-Penedo *et al.*, 2006). This interaction occurs at intestinal level. High zinc increases metallothionein expression in the intestine, which coats the lumen surface where it binds and sequesters copper (Bremner, 1979; NRC, 2001; Lee *et al.*, 2002; Suttle, 2012). The mechanism acts to decrease copper absorption through trapping copper within metallothionein in the intestinal mucosal cells. The copper is subsequently lost through faeces via apoptosis, when the enterocyte cells are sloughed into the lumen (Bremner, 1979; NRC, 2001; Lee *et al.*, 2002; Suttle, 2012). Since metallothionein has a stronger affinity for copper than zinc, high levels of metallothionein induced by excess zinc cause a notable decrease in copper absorption. The observed hepatic effect appears to due to a re-distribution of hepatic copper into metallothionein where hepatic zinc content is increased (Bremner and Marshall, 1974; Bremner *et al.*, 1976).

Under practical conditions, zinc may not contribute a major effect on copper absorption unless the diet contains excessively high levels, around twenty times the animals requirement (NRC, 2001).

1.11 Trace element interactions: Cadmium, selenium, and lead

Interactions have been reported between copper and a range of other elements. Some interactions which are less observed, and of less biological importance, include; cadmium, selenium and lead. A similar mechanism to zinc has been proposed for the effect of excess cadmium on copper. Diets containing >5 mg/kg can cause copper concentrations in liver to decline (NRC, 2001). It has been suggested that a cadmium supplemented diet is linked with the increased mucosal binding of copper (Davies and Campbell, 1977; Spierenburg *et al.*, 2013).

It has been debated if an interaction exists between copper and selenium at dietary inclusions ranging from 0.2-5 mg/kg DM. Researchers have found no interaction in cattle (Buckley *et al.*, 1986), while others have observed effects in sheep and goats (Thomson and Lawson, 1970; Hussein *et al.*, 1985; Hartmann and Van Ryssen, 1997). The nature of the interaction is also debated. A synergism has been observed in both elements (Hartmann and Van Ryssen, 1997; Van Ryssen *et al.*, 1998; Netto *et al.*, 2013). Possibly due to increased intestinal absorption though reduced metallothionein synthesis caused by selenium; creating the opposite effect to zinc and cadmium (Evans and Johnson, 1978; Chmielnicka *et al.*, 1983). An antagonism has also been suggested, although evidence remains anecdotal (Koh and Judson, 1987; Johnson *et al.*, 1997; Netto *et al.*, 2014). Other studies have found no association or detectable difference (Netto *et al.*, 2013). It has been suggested that factors including; sulphur concentration, age, health status, administration method and previous copper status may contribute to these variable findings (Van Ryssen *et al.*, 1998).

A potential interaction with lead at concentrations >100 µg/kg WW in the liver is also much debated. In cattle, some studies have described a negative correlation; where high copper predisposed low lead and vice versa. However, others have found no significant link, and further studies found a positive correlation where the presence of one element increased the other (López-Alonso *et al.*, 2002, 2004; Miranda *et al.*, 2005; Blanco-Penedo *et al.*, 2006; Spierenburg *et al.*, 2013).

1.12 Measuring copper status

Determining the copper status of an animal is not a simple process. There is no single definitive test currently suitable for this purpose (Laven and Livesey, 2009). Needle biopsies of the liver in live animals provide the most accurate estimates of overall copper status (Kincaid, 1999; Miranda *et al.*, 2010). However, they represent the longer-term copper status and previous diet, not the present functional copper status of the animal (Laven and Livesey, 2006). This invasive method also poses practicality and cost implications and is often unsuitable for routine use.

Blood sampling is much more cost effective and widely available, but the use of blood parameters only provides an approximate measure of copper status (Laven and Livesey, 2009). Measures of whole blood copper concentration include erythrocyte and plasma copper and have a role as a measure of medium-term copper status. Erythrocyte SOD can be sensitive to changes in copper status, but since erythrocytes have a relatively long life-span this measure is slow to respond to changes in copper status (Kincaid, 1999; Laven and Livesey, 2009; Laven, 2014). Over longer term studies, declines in erythrocyte SOD were found to be more closely correlated with declines in growth rate and increased mortality (Jones and Suttle, 1987).

Copper concentration in serum and plasma are considered to reflect the more recent changes in copper status (Laven and Livesey, 2006; Suttle, 2010). Despite measuring the same copper quantity, serum contains 5-30% less copper than plasma (Suttle, 2010). The difference is likely due to a greater sequestering of copper, as caeruloplasmin, into the clot (Paynter, 1982; Suttle, 2002; Laven and Livesey, 2006). This makes plasma copper concentration the preferred choice and is a useful indicator of copper status when hepatic copper is deficient (<281 µmol/kg DM) (Claypool *et al.*, 1975; Kincaid, 1999). Before this point, plasma copper concentration appears to be maintained by the mobilisation of copper from the liver, thus reducing its sensitivity to copper status beyond deficiency detection (Blakley and Hamilton, 1985; Laven *et al.*, 2007; Suttle, 2010; Dairy Co, 2012b; Dezfoulian *et al.*, 2012). Various factors such as recent vaccination, disease and even the onset of hypo-cupremia have been suggested to be able to produce an increase in plasma copper concentration reducing the reliability of the measure (Suttle, 2010).

In plasma and serum, ~70-90% of copper is associated with caeruloplasmin (Hosking *et al.*, 1986; Vulpe and Packman, 1995; Milne, 1998; Nevitt *et al.*, 2012; Safdar and Kor, 2014). Although, some is found in other enzymes and bound to proteins and amino acids (Laven and Livesey, 2006; Suttle, 2010). The use of serum caeruloplasmin activity has become a commonly used method of measuring blood copper status (Suttle, 2010). Copper-containing enzyme activity is considered a better indicator of metabolically active copper over its concentration (Milne, 1994; Dezfoulian *et al.*, 2012). However, caeruloplasmin is an acute phase protein; known to increase as part of the immune response to both chronic and acute infections and stress (Blakley and Hamilton, 1985); potentially resulting in elevated caeruloplasmin activity beyond that of the animal's copper status and creating difficulty in detecting elevated status from an acute phase response (Arthington *et al.*, 2003; Suttle, 2010). Despite this, it is still considered to be a reliable indicator of copper status in both the deficient (<15 mg/dl) and high-normal (35-45 mg/dl) ranges (Blakley and Hamilton, 1985; Linder *et al.*, 2003; Laven *et al.*, 2008; Dezfoulian *et al.*, 2012). Under practical conditions it is possible that caeruloplasmin activity offers little advantage over plasma copper concentration, due to a high correlation between the parameters (Laven and Livesey, 2004). The assay may also be susceptible to interference reducing the correlation between the parameters, but also reducing the accuracy of the diagnostic (Suttle, 2010). Copper supplementation elicits a marked elevation on caeruloplasmin activity after deficiency has been established, making this measure useful in the detection of blood responses to copper repletion treatments (Blakley and Hamilton, 1985).

The use of cytochrome c oxidase (CCO) activity in platelets and leucocytes may be able to reflect metabolically-active endogenous copper, since these cells have short life-spans. However, the required specific cell isolation is labour intensive and the enzyme highly labile; resulting in highly variable assay results (Milne, 1994). When measured in cattle and sheep CCO declined more slowly than plasma copper or caeruloplasmin, making it a less reliable measure (Kincaid, 1999). Fluctuations in this assay and in response to other physiological and biochemical factors makes the use of CCO as variable as caeruloplasmin (Milne, 1998; Stern *et al.*, 2007).

In the absence of a single, reliable measure for copper status the need to monitor at least several of these biochemical markers over a period of time, in preference to a single sample, is evident to give the most accurate assessment of copper status (Failla, 1999). Although, the accurate measurement of current clinical copper status still remains problematic even when multiple parameters are measured.

1.13 Measuring thiomolybdate status

Further complexity is added to measuring copper status through the potential detection of systemic copper sequestered by thiomolybdate. Copper complexed to thiomolybdate is no longer biologically available but may still be detectible by assay. High molybdenum diets can increase plasma copper through the detection of the copper-thiomolybdate complex, despite liver copper being depleted and caeruloplasmin activity being reduced (Smith *et al.*, 1968; Kincaid, 1999). Caeruloplasmin activity has been shown to diminish alongside the administration of thiomolybdate (Lannon and Mason, 1986). However, to further distinguish the effects of thiomolybdate and increase reliability of serum caeruloplasmin activity a plasma copper concentration ratio (Cp:Cu) can be used. This measurement is based on the hypothesis that the presence of thiomolybdate would affect caeruloplasmin activity more than plasma copper concentration (Laven and Livesey, 2009). Although, it is regarded as a reliable method of blood analysis for detecting a thiomolybdate problem, it has received criticism as an unreliable indicator of thiomolybdate exposure in cattle (Mackenzie *et al.*, 1997b; Livesey *et al.*, 2010).

A further method used for the detection of systemic thiomolybdate is the use of tricholoro-acetic acid (TCA) to precipitate copper-thiomolybdate from plasma (Kelleher *et al.*, 1983; Kincaid, 1999; Telfer *et al.*, 2004; Suttle, 2010; Bidewell *et al.*, 2012). In the absence of systemic thiomolybdate, copper remains in the TCA soluble fraction with negligible copper in the TCA precipitate (Mason, 1986a; Gooneratne *et al.*, 1989a). *In vitro* and field studies have determined that animals fed high concentrations of sulphur and molybdenum alongside high copper did not have TCA insoluble copper in their plasma (Dick *et al.*, 1975; El-Gallad *et al.*, 1983). However, when the copper content was reduced, and in animals which were fed high sulphur and molybdenum without high copper, TCA insoluble copper was present (El-Gallad *et al.*, 1983; Yuan *et al.*, 1988). The addition of molybdate without sulphur also gave no effect on TCA soluble copper confirming that the presence of TCA insoluble copper represents the presence of systemic thiomolybdate (Dick *et al.*, 1975). Despite this evidence, it has not been established that copper-thiomolybdate is the sole constituent of TCA-insoluble copper.

Requiring further validation before it can be used as an unequivocal measure of thiomolybdate presence (Laven and Livesey, 2005).

1.14 Conclusion

Copper status and requirement in ruminants is difficult to assess and define. Multi-faceted issues surround the decision to supplement and with what copper source. The interactions which take place are unique to ruminants, yet copper imbalance accounts for one of the widest reported mineral problems. Further investigation into the mode of action of the antagonists and the effects of different copper sources on their action is integral to improving the copper status and welfare of ruminants worldwide.

1.15 Hypothesis & Aims

The overarching aims of this work were to try and determine how copper moves through digestion. To investigate the interactions of copper and its antagonists for a range of copper sources at different regions of the ruminant digestive tract. To elucidate the mode of iron-sulphur antagonism and to investigate the relationship between antagonist routes. A secondary aim was to assess the understanding of copper-related terminology and problems, across the farming community through a national survey. The hypothesis for study is described in Figure 3.

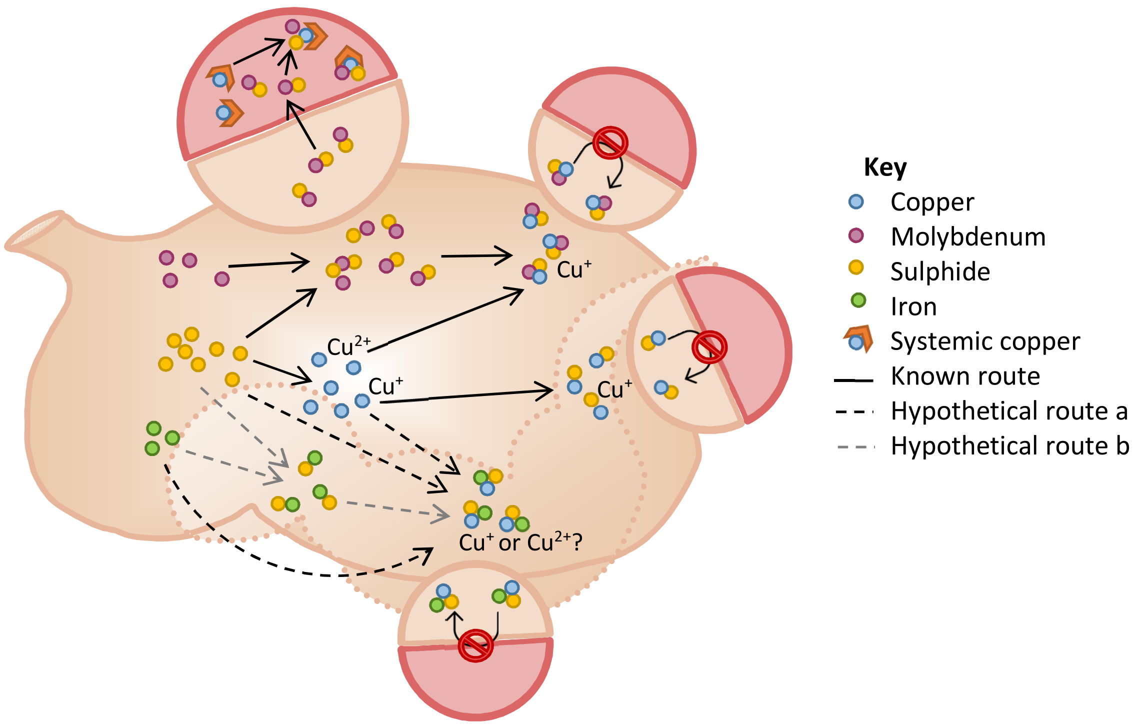

Figure 3: High affinity antagonist pathway molybdenum-sulphur-copper first forms thiomolybdate in the rumen. Where copper is available thiomolybdate will bind copper making it un-absorbable. If copper is not available thiomolybdate will enter systemic circulation and bind with biological copper compounds (Spears, 2003; Gould and Kendall, 2011). Lower affinity sulphur will bind with copper creating insoluble copper sulphide which is lost through egestion (Bird, 1970; Suttle, 1974a; Spears, 2013). Unknown interaction iron may bind with sulphur and copper creating an unknown un-absorbable complex, or iron may bind to sulphur creating iron sulphide which later dissociates and forms copper sulphide (Suttle and Peter, 1984; Rosa *et al.*, 1986; Gould and Kendall, 2011).

2.0 Copper supplementation: Perception and practice in UK farming

2.1 Introduction

In this chapter the farmer's perspective of the 'copper conundrum' will be investigated. Section 1.6 discussed the complexities which surround the decision to supplement copper on a farm, herd or flock basis. Research has found that very few UK farms are able to balance their animal's copper needs with their supplementation strategy (Livesey *et al.*, 2002; Dairy Co, 2012b; Sinclair and Atkins, 2015). Investigation into perception and understanding of copper related problems have focussed on industry professionals (Black and Kendall, 2010; Kendall and Bone, 2019). Their understanding is important as it provides the knowledge that informs the farming community. However, little is known about know well farmers understand this information and associated terminology or what guides them in their supplementation strategy.

Black and Kendall, (2010) surveyed copper knowledge in veterinary and non-veterinary advisor groups; finding there was inconsistency and very little consensus on the prevalence and cause of copper-responsive disorders; defined as any clinical condition through which clinical signs can be alleviated through increasing copper supply. A little over half the respondents correctly identified primary copper deficiency as rare in the UK (58% vets, 62% advisors) while the remaining proportion considered it common. The cause of copper deficiency also divided opinion 68% vets and 72% of advisors felt it was antagonist driven, although these respondents were roughly 50% divided between those who considered it rare and those who considered it common, furthermore, 24% vets and 43% advisors felt molybdenum was responsible for the problem. The lack of consensus between those in a position of mentorship to farmers likely leads to mixed messages. Where previous research has included reference to farmer knowledge or understanding, the key demographic have not been extensively questioned and the researcher's observations have relied on anecdotal evidence and personal experience (Black and French, 2000; Livesey *et al.*, 2002; Bidewell *et al.*, 2012; Bowyer, 2016; Hunt, 2016).

In order to fully appreciate the current position of farmers and determine how the disparity in veterinary and advisor understanding impacts them and their supplementary decision making, a national survey was designed to assess; current supplementation strategies, understanding of copper related disorders, their perceived prevalence, and their terminology.

2.2 Aims & Objectives

The aim of this work was to survey UK ruminant farmers from across dairy, beef and sheep enterprises in order to identify current copper supplementation strategies and ascertain the motivation behind supplementation routines. A second aim was to find out the level of perceived and actual understanding of copper related disorders and their terminology.

2.3 Materials & Methods

2.3.1 Survey design

An online survey was designed using Jisc (Jisc, Bristol UK) and links to the survey were distributed using social media (Facebook Inc. Massachusetts, USA) and e-newsletters circulated by the Agriculture and Horticulture Development Board (AHDB), Hybu Cig Cymru (HCC), National Beef Association (NBA) and the National Sheep Association (NSA). The survey was open for a period of 3 months and was continually promoted using these outlets during this time. Participation was incentivised through the chance of winning a farm mineral audit. A combination of both quantitative and qualitative data was gathered though the use of a range of simple closed questions, multiple choice questions, scale and rating questions and free text answers (full questionnaire in Appendix I). The survey was split into three sections. The first collected data on the type of farming enterprise, husbandry and supplementation information. Logic pathways were used in this section to allow farmers to complete only the sections relevant to their farm (Figure 4). The second section gathered information relating to perception of copper related problems and terminology. The final section gathered demographic data on geographic location and experience.

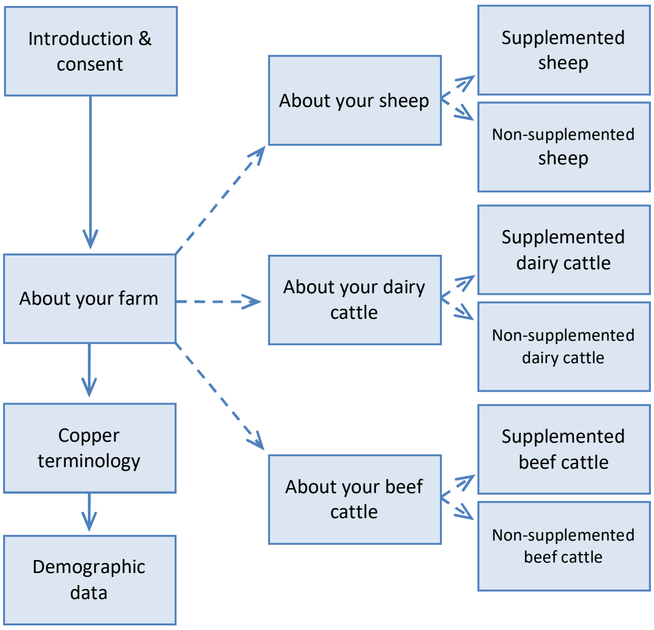

Figure 4: Survey sections and use of logic pathways. Solid line denotes sections completed by all participants, dashed line shows sections which used logic pathways to allow completion only where participants indicated they were relevant.

2.3.1.1 Inclusion criteria

Participants were required to be located within the UK (excluding Republic of Ireland) and currently farm cattle or sheep. No minimum number of animals was required.

2.3.2 Pilot study

The survey was sent out in pilot form to a small number of willing representatives from beef, dairy and sheep enterprises. These respondents were asked to complete the survey and asked if there were any areas of ambiguity or if there were any changes and additional options they would make. The pilot data was suitable for analysis as only minor changes were suggested by the pilot group. Minor changes to clarify wording were made prior to distribution of the final version.

2.3.3 Data capture

Data was gathered and analysed in line with current GDPR legislation and University of Nottingham policy. Participants were asked to confirm their agreement to the data use, storage and purpose of the survey and that they were over 18 years old before they undertook the survey. Participants were able to withdraw from the survey at any point and only questions required for consent and logic pathways were marked as mandatory.

2.3.4 Data management and analysis

Anonymous raw data was downloaded and processed using MS Excel (2013, Microsoft, Washington, USA) for grouping, coding and analysis. Statistical analysis of data was performed using Minitab v.18 (Minitab, Pennsylvania, USA) for Chi squared test and ANOVA of numerically coded data. Likert scale data was analysed using parametric testing, which has been demonstrated to be valid and robust on this data type (Carifio and Perla, 2007; Norman, 2010; Boone and Boone, 2012; Wigley, 2013; Wadgave and Khairnar, 2016). In all relevant tests, an α value of $P<0.05$ was used as the cut-off for significance. Subsequent increased levels of significance were determined at $P<0.01$, $P<0.001$ and $P<0.0001$.

2.4 Results

2.4.1 Exclusions

A total of 254 respondents completed the survey. One respondent was not UK based and was deleted prior to analysis. Some respondents did not answer every question within the survey. The data from these respondents was included where relevant and where questions were incomplete their data was omitted and adjusted accordingly. In the reported data n numbers in parenthesis show the number of responses analysed.

2.4.2 Demographic data

Respondents were asked if they were personally responsible for nutritional decisions on the farm to establish the relevance of the data gathered to on farm practice, 99% (n=250/253) confirmed that they were. At the end of the survey, they were asked to provide the first part of their postcode to determine the breakdown of respondents across the surveyed region (Figure 5).

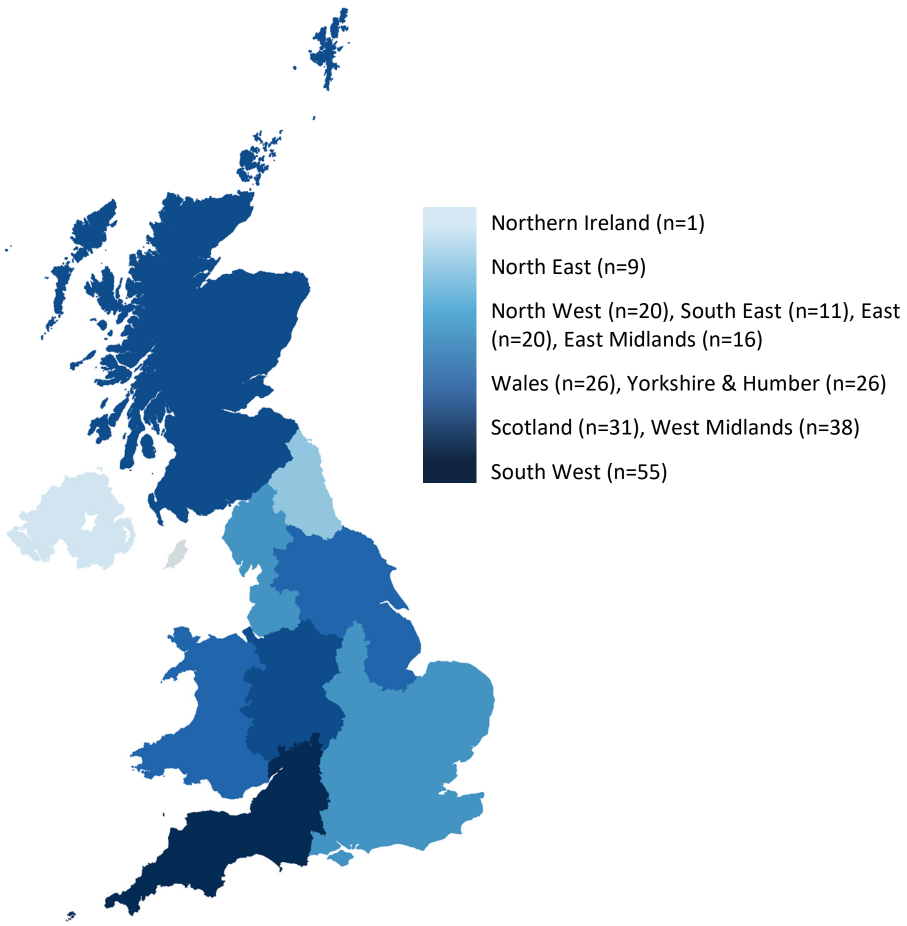

Figure 5: Regional distribution of respondents. Colour coded from darkest to lightest to indicate respondent density by region.

The majority (48%, n=121/253) of farmers that completed the survey kept both beef and sheep, followed by 27% (n=68) who farmed just sheep, 12% (n=29) who farmed beef and 6% (n=15) who farmed dairy. Dairy cattle in combination with beef or sheep represented 6% (n=15) of respondents. Only a small percentage (2%, n=4) kept all three enterprises. Enterprises were varied in terms of herd/ flock size. Mean dairy herd size was 317 (±217, range 65-1200), mean beef herd size was 171 (±264, range 4-2500) and the mean sheep flock size was 601 (±889, range 3-8,000). Farmers also varied in their experience from 1-60 years with a mean of 23 (±14) years farming experience. Each of respondents rated their own copper knowledge from ‘none at all’ to ‘excellent’ (Table 1).

Table 1: Breakdown by percentage and number of respondents for farmer’s own rating of their copper knowledge.

	Percentage (%)	Number of respondents (n)
‘no knowledge at all’	8	20
‘a little knowledge’	29	72
‘some knowledge’	47	120
‘sufficient knowledge’	12	29
‘good knowledge’	4	10
‘excellent knowledge’	0.8	2

Across all respondents 93% (n=223/251) were interested in learning more about copper related problems.

2.4.3 Husbandry and supplementation strategies

Dairy herd composition was calculated for breeds which contributed ≥50% of the herd. Most dairy respondents (72%, n=23/33) had herds which were made up of Holstein Friesian cattle and crosses of this breed. Jersey cows were the next represented breed, accounting for 16% (n=5) of dairy respondents. Most of the dairy herds were housed over winter (74%, n=25); defined as housing the animals for more than 2 consecutive months between November and January for a typical year. Only 9% (n=3) of dairy respondents indicated their animals were permanently housed. The majority (47%, n=16) of dairy farmers responded that they supplemented their herd with a general mineral supplement that was not specifically for copper. Respondents who supplemented their dairy cattle with copper accounted for 24% (n=8) and the remaining 27% (n=9) stated they did not supplement with copper.

Beef herd composition was much more varied. Breeds were counted where they contributed ≥30% of the herd. The most common breeds were Limousin and Aberdeen Angus, both 23% (n=37/165) of respondents, followed by Hereford (15%, n=24) and Charolais (9%, n=15). Most

(76%, n=126) beef respondents indicated that their animals were housed over winter and only 8% (n=14) were never housed. Similar to dairy cattle, the majority (58%, n=96) of beef farmers stated they supplemented their herd with a general mineral, non-copper specific. A similar percentage to dairy (25%, n=41) also stated they supplemented specifically with copper. The remaining 17% (n=28) stated they did not supplement their animals.

Sheep breeds were also varied in flock composition. Breeds were counted where they contributed ≥30% of the flock. The most represented breed was Texel and Texel crosses (28%, n=55/197), followed by Mules (21%, n=41), Suffolk (9% n=17), Lleyn (8%, n=16) and Welsh mountain (7%, n=14). Most sheep flocks (48%, n=94) reported only housing during spring. Spring was defined as housing for at least one month between February-April. A further 12% (n=24) reported housing their flocks over winter and 30% (n=59) were never housed. Similar to cattle, the majority of sheep farmers (42%, n=83) also provided general mineral supplementation, not specifically copper. Those who supplemented with copper accounted for 31% (n=62) and 26% (n=52) did not supplement.

Across both species and all enterprises two key reasons for choosing not to supplement with copper, or to supplement non-specifically, were highlighted. The reasons with most agreement were an 'absence of deficiency signs' and 'providing a multi-mineral containing copper'. Figure 6 shows the breakdown of responses for the factors ranked most important across all enterprises.

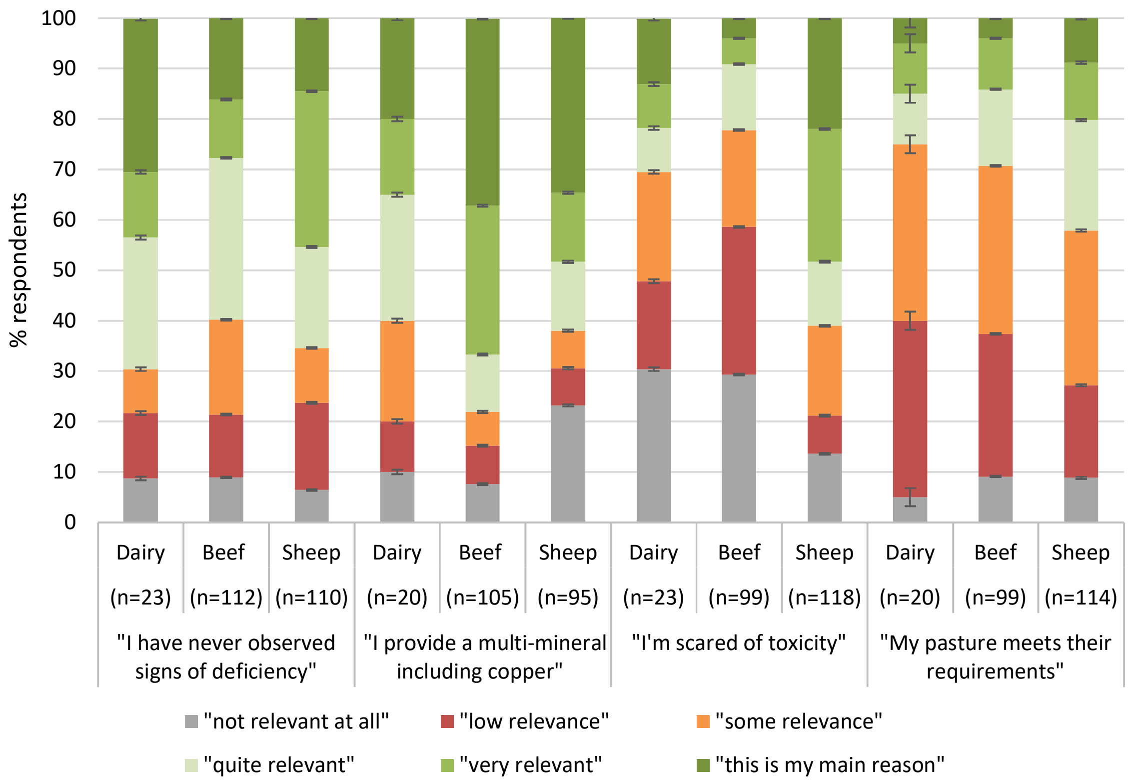

Figure 6: Percentage breakdown by enterprise and relevance score for reasons to refrain from specific copper supplementation. Number of respondents (n) shown in brackets. Error bars denote standard error of the mean ($\sigma\bar{x}$) using numerically coded responses; 'not relevant at all' 1, 'low relevance' 2, 'some relevance' 3, 'quite relevant' 4, 'very relevant' 5, 'this is my main reason' 6.

Farmers who considered an absence of deficiency signs as a key factor in their supplementation decision making was (P<0.01) different between enterprises. On average, dairy enterprises scored this factor higher ($\tilde{x}$4 ±1.9 'quite important') suggesting that this factor is more relevant to them than those who farm beef and sheep ($\tilde{x}$3 ±1.9 'some importance'). The importance of providing a multi-mineral supplement containing copper was also (P<0.01) different between enterprises. This factor was scored as less important to sheep farmers and of highest relevance to beef farmers ($\tilde{x}$2 'low importance' and 5 'very important' respectively). The potential for toxicity was considered 'not relevant' by ~30% (n=36) of cattle farmers, while sheep farmers considered this factor to be the most relevant (n=57 'very' or 'main reason'). This was different (P<0.0001) between enterprise and species; indicating that concern for toxicity is more ubiquitous with sheep than cattle. This was echoed by the overall percentage who agreed completely (43%, n=107), or a little (30%, n=75), that cattle are more tolerant to high copper than sheep. Factors which were considered to have 'no relevance' by the majority of respondents across all enterprises included; the cost and labour required for supplementation, providing a total mixed ration which met requirements, only supplementing when animals were housed or at pasture, and having previously supplemented but deciding to stop.

The reasons for supplementing copper were similar across enterprise and species. Figure 7 shows the factors ranked most important to the majority of respondents across all enterprises.

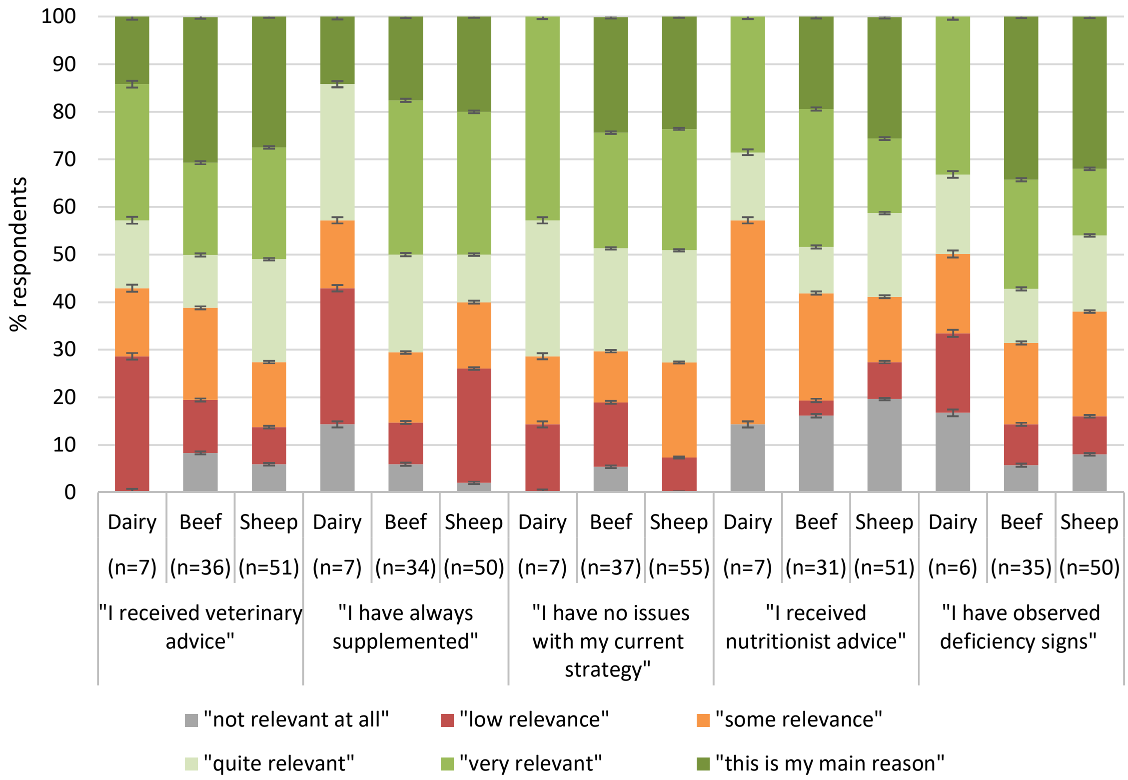

Figure 7: Percentage breakdown by enterprise scored by relevance for reasons to provide specific copper supplementation. Number of respondents (n) shown in brackets. Error bars denote standard error of the mean for numerically coded responses.

Continuing to supplement by tradition was considered the most relevant factor across all enterprises. This factor appeared more relevant to beef and sheep farmers than dairy farmers, but was not significantly different. Supplementing on the basis of veterinary advice appeared slightly more relevant than nutritionist advice across all enterprises. Those farmers who felt they had 'good' understanding of copper problems considered this factor more important ($P<0.05$) than those with 'sufficient' understanding. Although, there was no significant difference between enterprise, veterinary advice was only given as a main reason by beef and sheep farmers (21%, n=11 and 15%, n=14 respectively). Observing the signs of clinical deficiency was also only a main reason for beef (30%, n=12) and sheep (26%, n=16) farmers. Factors which were considered 'not applicable' by the majority of respondents across all enterprises were; trying to remedy mystery health and fertility problems, influences from mineral sales representatives and magazine advertisements, and only supplementing when the animals were housed or at pasture. Despite the low overall importance, sheep farmers were more likely than beef farmers to supplement for an unknown health problem ($P<0.05$). Beef farmers were more likely to supplement copper only at housing in comparison to sheep farmers ($P<0.05$) and sheep farmers were more likely to supplement copper only when their animals were at pasture in comparison to dairy farmers ($P<0.05$).

Most (80%, n=89/111) farmers used a combination of copper supplements. The most popular were boluses (77%, n=86), free access mineral licks (64%, n=71), premixes (47%, n=52) and drenches (35%, n=39). Injections and pasture dressing were used much less in comparison (12%, n=13 and 5%, n=6 respectively). Among sheep farmers, boluses were the most popular (81%, n=50/62) followed by mineral licks (58%, n=36/62). Beef farmers also preferred these methods (80%, n=33/41 mineral lick, 76%, n=31/41 bolus). Whereas, dairy farmers favoured boluses (63%, n=5/8) and mineral premixes (50%, n=4/8). Around half of the farmers varied their supplementation strategy by production stage. Strategies were differentiated for breeding animals and young stock across dairy and beef. While, ewes and lambs were differentiated in sheep. It was interesting to note that four sheep farms also differentiated by breed.

Around 50% of all farmers (n=53/111) tested their soil prior to making their supplementation decisions. Slightly more (55%, n=61/110) also tested their herbage and 63% (n=70/110) also blood tested their animals. Interestingly, only 12% (n=13/108) tested liver samples prior to decision making. Finally, 3% (n=3/105) tested other tissue samples such as kidney and 5% (n=5/104) tested urine or milk.

When asked to predict the percentage of their herd or flock which would be copper deficient without supplementation the results ranged from 1-100% ($\bar{x}$ 61±32, $\tilde{x}$65), with no significant difference between enterprise, experience or understanding.

The majority (56%, n=140/251) of farmers said they felt under no pressure to supplement their animals with copper. A further 18% (n=44) felt under low pressure and the same percentage under some pressure. Only 4% (n=10) felt under a lot or immense pressure. There was no difference in pressure felt across enterprise, understanding or experience. Factors which were considered to have no pressuring effect by the majority of respondents were; magazine advertisements, family, farm workers, and neighbouring farms.

Despite 43% (n=109) of farmers stating nutritional advisors had no or little pressuring effect there was an ($P<0.01$) effect of understanding on this factor. Those who rated their understanding of copper problems as ‘excellent’ felt under a lot of pressure, and those who had ‘no understanding’ felt under low pressure from advisors to supplement. All other understanding levels found them ‘not at all pressuring’. Vets were considered to have no or little pressuring effect by 49% (n=124) of all respondents. However, similar to nutritional advisors there was an ($P<0.01$) effect of understanding. Those who rated their understanding as ‘excellent’ felt under a lot of pressure and those who felt they had ‘good understanding’ and ‘no understanding’ both felt under ‘some pressure’ in comparison to the rest. Both nutritional advisors and vets were considered the most pressuring out of all the potentially influential factors, as a total of 14% (n=34) of respondents rated them as highly to immensely pressuring.

2.4.4 Copper terminology and understanding

To demonstrate understanding of copper related problems farmers were asked to identify various factors as causes of copper related problems. Figure 8 shows the breakdown by percentage respondents for their answers.

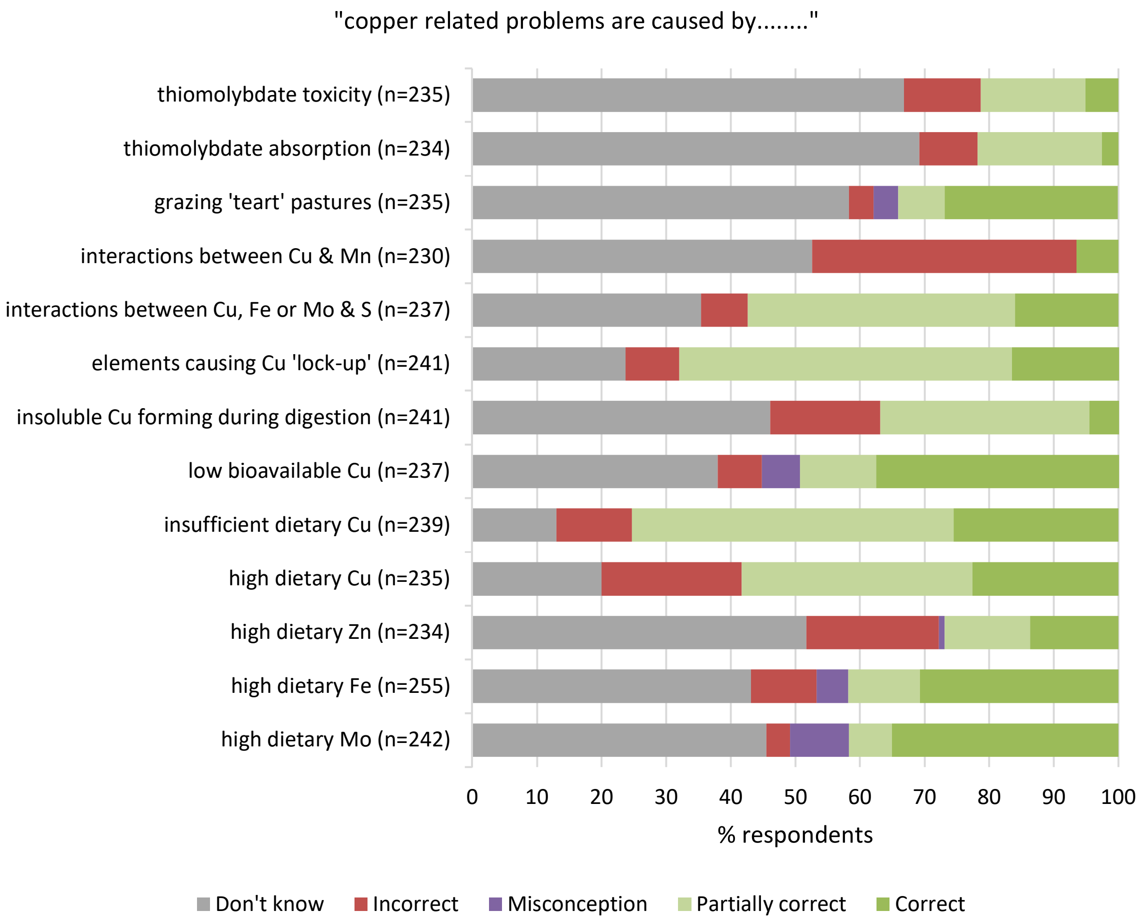

Figure 8: Percentage breakdown for answers identifying factors potentially responsible for copper related problems by all respondents.

A high percentage (>40% n=~100/~230) of respondents returned a 'don't know' answer for the majority of potential causes; suggesting that specific knowledge on causal factors was not well understood by respondents. Surprisingly, only ˜24% (n=~55) correctly identified high or insufficient dietary copper as causes of copper related problems. Although this rose to 58% (n=137/253) and 75% (n=180/239) respectively when partially correct answers were included;

indicating simple provision of copper is not well understood as a causal factor. Interestingly, more farmers correctly identified copper of a low bioavailability as a causal factor than those able to identify insufficient provision. Most (75%) of the farmers who felt they had 'some understanding' answered this question correctly; which was ($P<0.05$) better than all other groups.

In general, farmers appeared unfamiliar with the specific role of molybdenum in copper related problems. Very few (4%, n=18/469, total from 2 questions) were able to correctly identify the role of thiomolybdate. Those who rated their understanding as 'good/excellent' were much more likely ($P<0.05$) to correctly identify thiomolybdate toxicity as a causal factor. However, this was not the case for thiomolybdate absorption. A higher percentage (16%, n=38/237) were able to identify the role of sulphur in both the iron and molybdenum antagonism, which was also ($P<0.0001$) better identified by those with 'good' understanding. Surprisingly, a much lesser percentage (5%, n=11/241) identified the formation of insoluble copper as a causal factor. Where the terminology was changed to copper 'lock-up' farmers seemed better able to identify this same outcome (17%, n=40/241) and appeared more familiar with the term; as all levels of understanding beyond 'not at all' were ($P<0.0001$) better at identifying this cause, and those with 'good' understanding were 90% correct. Farmers were generally able to identify iron and molybdenum, including teart pasture, as causal factors ($\bar{x}$ 27%, $\bar{x}$n=64/242). However, there was a notable percentage (5-10%) who potentially overlooked the participation of sulphur in this interaction. Those who had 'very little' or 'no understanding at all' were ($P<0.0001$) worse at identifying these factors, with ≤10% correct. High zinc was identified as being partially able to cause copper problems by 14% (n=32/234) of respondents with no significant difference in ability to identify it by any group. Only 7% (n=15/230) correctly identified that manganese was not a copper interacting element. Notably, 41% (n=94/230) incorrectly declared this as an interacting element equating to the highest incorrect score. Those with 'good' understanding were 25% correct; which was ($P<0.01$) better than those with 'very little understanding' and 'no understanding' who were all unable to identify the correct answer.

To further investigate understanding, farmers were asked if they were familiar with a series of key terms and to provide a definition where they felt able (Figure 9). Reference definitions used are outlined in the glossary of terms and definitions on Page *xxi- xxii*.

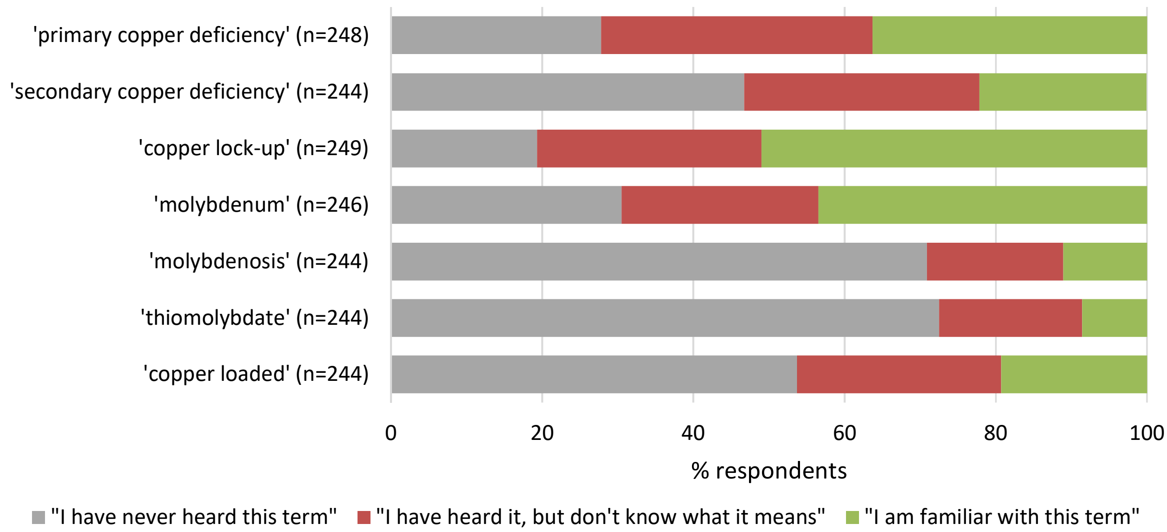

Figure 9: Percentage of total respondents and their self-assessed familiarity with copper terminology.

The terms respondents were most familiar with included; 'molybdenum' and 'copper lock-up' around 50% (x̄n=117/248) of respondents indicated they were familiar with these terms, followed by 36% (n=90/248) who were familiar with 'primary copper deficiency'. Terms much less familiar were 'molybdenosis' and 'thiomolybdate'. Only 10% (x̄n=24/244) felt these were familiar terms. The respondents who were familiar with the terms were asked to give a definition to help assess the level of familiarity and use (Figure 10).

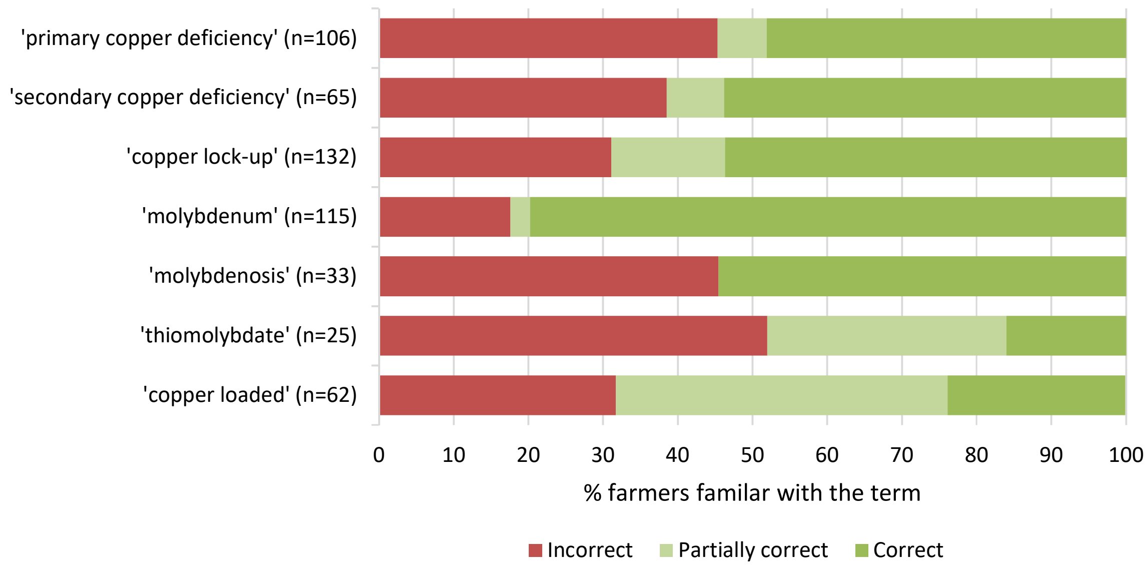

Figure 10: Percentage correct, partially correct and incorrect definitions from those who considered themselves familiar with the copper terminology.

The more familiar term 'molybdenum' was defined accurately by 80% (n=92/115) of those accustomed with it. The definitions given for this term were succinct and relatively unvaried between correct respondents. 'Copper lock-up' and 'primary copper deficiency' were lesser defined ˜50% ($\bar{x}$n=61/119) of those familiar with these terms were able to give an accurate definition. The most common inaccurate definition was that 'lock-up' referred to a lack of availability, or absence, of copper in the soil. Primary copper deficiency had more varied incorrect definitions. Common misconceptions were that, again, the deficiency was within the soil, or that the term related to the presence or absence of clinical signs. Interestingly, 13% (n=14/106) of those familiar with primary copper deficiency thought it referred to the main mineral the animal was deficient in. Despite a lesser number of respondents familiar with secondary copper deficiency it was similar in ability to define. Around half (54%, n=35/65) of those familiar with the term could give an accurate definition. Incorrect definitions for this term were similar to that of primary deficiency, with respondents stating it related to an absence or presence of clinical signs or a deficiency within soil or feed. The most common misconception reported by 6% (n=5) was that copper was deficient, but that this was not the main mineral the animal was deficient in. 'Molybdenosis' was not considered a familiar term by many respondents. However, 55% (n=18/33) of those who felt they knew it were able to give an accurate definition, which in most cases was clear and succinct. The incorrect answers most commonly (27%, n=9) quoted molybdenum's influence on copper availability rather than the direct effects of an excess of molybdenum. The lesser known terms 'thiomolybdate' and 'copper-loaded' were correctly defined by 16% (n=4/25) and 24% (n=15/62) respectively, which increased considerably to 68% (n=43) where partially correct answers for 'copper-loaded' were included. For 'thiomolybdate' the incorrect answers were very varied and appeared to be guesses. Whereas 'copper-loaded' was incorrectly defined as providing a high copper intake, from various sources, and worryingly, by a small number of respondents as an ideal amount that should be aimed for.

Finally, farmers were asked to estimate the percentage of the UK population for dairy, beef and sheep which were above and below ideal copper status. Means for every enterprise for both high and low copper fell between 30-40% (±21-23) with no significant effects of experience, enterprise or understanding, suggesting these were guesses by the majority of participants.

2.5 Discussion

The survey represented ~0.3% of UK ruminant farms (DEFRA, 2018). The responses in this survey suggest that ~0.2% of dairy, ~0.9% of beef and 0.5% of sheep farms were represented which provides a margin of error of ~6% (Bartlett *et al.*, 2001). Although, farmers were distributed across the UK, regional analysis was not appropriate due to varying numbers in each region and only one respondent from Northern Ireland.

The majority of farmers across all enterprises supplemented with a general mineral supplement, non-specific for copper. The complex and diverse nature of trace mineral supplementation may mean that farmers find it more convenient to provide a mineral supplement which is designed to meet the broad spectrum needs of their animals. The convenience of such products may contribute to their popularity of use. Data regarding the level of concern for the animal's copper status was not gathered in this study, although it may have been interesting to investigate any correlation between specific copper supplementation and concern for copper status. A wide range of answers were given to estimate the percentage of flock or herd which would become copper deficient without supplementation. This, in combination with the reasons given for cessation of copper supplementation suggested, in general, supplementation was not a targeted or tested approach and was provided purely as part of routine husbandry.

Farmers tended to supplement using boluses, free-access mineral licks and premixes by preference, in line with previous research across cattle and sheep which showed similar preferences for these products (Black and Kendall, 2010; Dairy Co, 2012b; Clarkson *et al.*, 2017; Hession *et al.*, 2018). These products are often marketed as long-lasting and reliable, both of which are appealing to farmers. Free-access mineral licks also have the advantage that they are able to be used while the animals are at pasture without the need for handling. The work by Hession *et al.*, (2018) highlighted ease of use, labour, and cost, as important factors influencing choice of supplementation method. However, the efficacy and reliability of these products is debated. Free-access minerals are known to have variable individual consumption and do not provide a known dose to each individual. While most (75-80%) animals have been shown to visit and use free-access minerals the intake of mineral can range notably from as little as 20 g up to ~400 g per feed (Tait and Fisher, 1996; Garossino *et al.*, 2005). Despite anecdotal evidence, it appears that animals are unable to regulate their free-access mineral intake in relation to requirement, making them a highly variable source of copper (Burritt, 2012). Drenching, especially to correct deficiency, is considered somewhat unreliable due to the variation in oesophageal groove and degree of rumen by-pass (Suttle, 2005). Some have been demonstrated to be effective for only 1-2 weeks (Kendall *et al.*, 2000). Drenching can increase measures of copper status, but has been shown to last only a short period of time where molybdenum (>5 mg/kg DM) is present in forage (Ivan, 1993; Peers and Phillips, 2011). Using a drench ensures that each animal receives an exact dose. Although, the administration of the drench requires regular handling and, if intended as long-term supplementation, a high copper tolerance (Kendall *et al.*, 2000). Boluses are considered to be more reliable long-term supplements which also provide an exact dose. Although, there are concerns about dissolution rate, and when tested, some boluses delivered results no better than controls (MacPherson, 1985). Newer boluses have been designed to eliminate these issues although dissolution ranging from 141-507 days have been reported (Kendall *et al.*, 2001).

Around half of farmers differentiated their supplementation strategy by production stage. The most commonly reported difference was providing different supplementation to young stock and breeding stock. This was described as providing additional copper at drying in dairy. Providing calves with additional supplementation in beef, and providing more copper to ewes, especially around breeding and lambing on sheep farms. The few sheep farmers who differentiated by breed all mentioned that they avoided supplementing the breeds deemed more copper sensitive. Differentiated strategies appeared to be conducted through tradition, occurring at key events or with changes in management. Only one farm mentioned routine blood testing and changing supplementation in line with the results. Another farm stated they differentiated by withholding supplementation and if health declined, providing it again at a lower dose. Both of these were sheep farms which may also be linked to concern for toxicity in this species. The use of differentiation by production stage is an indicator that farmers are aware of changing copper requirements through production. However, the large number that appear to continue their strategy without routinely checking input and impact on animal status may mean that these strategies are not as effective as intended (Kendall and Bone, 2019).

Both the presence and absence of deficiency signs were popular factors considered by the respondents of this study in their decision to supplement copper. The clinical signs associated with copper related disorders are wide ranging (discussed in Sections 1.2.1 and 1.2.2). Many of the signs are non-specific and largely unnoticeable in marginal cases (Judson and McFarlane, 1998). Where animals are extensively kept, like those in the current survey that only housed over winter or spring, or where animals are not routinely handled, it is potentially difficult to determine if this measure can be a useful reflection of the animal's copper status. Especially since it has been demonstrated that animals can have both low and toxic copper status without showing clinical signs (Suttle *et al.*, 1970; Mills *et al.*, 1976; Bremner *et al.*, 1987). The accurate interpretation of subtle clinical signs can also be problematic where more than one mineral is deficient, or where deficiency has impacted immune function and clinical signs of disease or parasitism conceal the underlying factor (Suttle and Jones, 1989; Judson and McFarlane, 1998). Additionally, a number of respondents referred to characteristic clinical signs such as de-pigmentation and neonatal ataxia which are often associated with severe copper deficiency, and in the case of de-pigmentation are arguably better associated with thiomolybdate toxicity (Suttle *et al.*, 1970; Mills *et al.*, 1976; Bone, 2010), discussed later in Section 9.2. This suggests that despite its popularity, relying on observation of clinical signs may not be a reliable tool for supplemental decision making.

Many respondents used a multi-mineral including copper. Respondents were not asked if they calculated the total copper input from all sources or how they checked they met their animal's requirements. This information would be interesting to determine how many, if any, calculate this and what methods are used to calculate copper input of the ration. However, it is likely that this is not a common process as Livesey *et al.*, (2002) found farmers lacked knowledge of their animal's copper intake and work by Sinclair and Mackenzie (2013) and Dairy Co (2012) found more than half of UK farms supplemented well above requirement. This is also supported by the work of Kendall *et al.*, (2015) who found a high percentage of UK dairy cattle had elevated liver copper.

The risk of toxicity was most important to sheep farmers, which was supported by them being less likely to use a multi-mineral that included copper. This finding was also in line with previous research by Clarkson *et al.*, (2017) who found that most UK finishing lambs were 'normal-low' in copper status, although, the previous work did not gather data on specific copper supplementation in these animals. Caution regarding toxicity in the present study may have been linked to the large proportion of participants who farmed Texel and Texel crosses; a breed which is known to be more copper sensitive (Menzies *et al.*, 2003; Price, 2016). It was interesting to observe that one farm kept North Ronaldsay sheep (12% of flock) and mentioned their susceptibility to toxicity but they only felt they had 'some' understanding of copper and failed to complete the questions on causation and terminology. The vast majority of farmers felt that cattle were more tolerant to copper than sheep. While this is not incorrect, it does appear to lead to a false perception of high tolerance in cattle (Clarkson *et al.*, 2019). Only

~30% of cattle farmers were concerned about toxicity despite the likelihood that they are receiving more than their requirement (Dairy Co, 2012b; Kendall *et al.*, 2015).

Continuing to supplement by tradition, through either having always done so or having no issues with the current strategy, were commonly reported in the current work. Hession *et al.*, (2018) found 20% of Irish sheep farmers also supplemented through tradition and a further 13% from experience. For those who supplement by tradition, it is likely that these strategies remain unchecked for their efficacy from year to year and subsequently may not be an optimal approach to providing supplementation to current livestock.

Nutritional and veterinary advice were some of the most reported reasons to supplement in the current study. A number of respondents also indicated their individual reasons for doing so in line with veterinary guidance and small number quoted nutritional advisor's guidance. In contrast, only 13% of Irish sheep farmers based their supplementation decisions on veterinary advice and 11% on guidance from nutritional advisors (Hession *et al.*, 2018). Although, these are professionals in this area, previous work has found that their guidance is not always consistent or reliable (Black and Kendall, 2010), and a notable number of nutritional consultants overlook key copper inputs before formulating feeds (Samuelson *et al.*, 2016).

Despite pressure from various sources (discussed in Section 1.6.1), the farmers in the current survey said they did not feel under pressure from them. It was interesting that those with 'good' and 'no understanding at all' felt more pressure to supplement from nutritional advisors and vets. Perhaps, those who feel they have better knowledge are more aware that they are being pressured and those who feel they have less knowledge feel more vulnerable.

Half the respondents who supplemented their animals with copper tended to base their decision making around soil, herbage and blood analysis. Although, worryingly ~15% of respondents across all enterprises did not base their decision on any form of diagnostic. The results found by Hession *et al.*, (2018), indicated a small number (22%) of sheep farmers based their supplementation decisions on laboratory analysis including; soil, herbage, blood or tissue analysis. Further distribution of how analysis was used was not studied. In the present work the majority (80%) of sheep farmers were found to base their decision on analysis of some form. However, only those who indicated they supplemented for copper were asked if they conducted analysis which may lead to the disparity between studies.

Soil and forage tests are not considered reliable measures for trace mineral status when analysed in isolation (Judson and McFarlane, 1998). In the present study, ~50% of farmers analysed both soil and forage and just two farmers reported analysing soil without any other type of analysis. Soil may constitute up to 10% of diet in grazing animals (Laven and Livesey, 2004). As such, the contribution of soil to total copper, and its antagonists, intake is important (Laven and Livesey, 2005). However, concentrations of minerals in the soil do not directly correspond with the nutrient composition of the herbage grown there (Ben-Shahar and Coe, 1992; Akhtar *et al.*, 2007; Dickson, 2016). Few soils express all 17 essential elements in the correct ratios and requirements for both plants and animals (Boom, 2002). Seasonal changes are known to effect the mineral concentration of the forage and in some cases these seasonal patterns can be used as a predictive tool to advise supplementation strategy (SFC, 2014; Clarkson and Kendall, 2018). Soil is only considered of use in copper deficiency diagnosis; as an initial tool to delineate areas of grazing where molybdenum is sufficiently high to create a potential problem (Suttle, 1991; Laven and Livesey, 2005), although, it not recommended as a single diagnostic even in this instance (Suttle, 1991). Pasture analysis is considered useful where animal analysis confirms a copper deficiency. Mineral requirements in pasture can be in excess of animal requirements for minerals such as sulphur and iron (Boom, 2002). Analysis of pasture or forage which constitutes the animal's primary diet can allow for a more effective determination of the cause of copper deficiency; through either an entire lack of copper provision, or the inclusion of antagonistic elements inhibiting copper availability (Suttle, 1991; Judson and McFarlane, 1998). Establishing the cause of deficiency allows a more effective supplementation choice to made (McFarlane *et al.*, 1990).

Due to the potential for elemental interaction and genetic variation in livestock, dietary analysis should not be used in isolation but should be supported by analysis of animal parameters; especially liver copper concentration (Laven and Livesey, 2004, 2005). Where pasture or forage samples are used in conjunction with animal samples, the risk of mineral problems arising can be more effectively ascertained (Judson and McFarlane, 1998). Soil and forage analysis is considerably advantageous to farmers over animal samples due to the ease of collection and lower analysis costs (Judson and McFarlane, 1998). In the present study, ~30% of farmers analysed both inputs and at least one animal parameter. More than half the farmers in the present study used blood sampling as a supplementation decision making tool. While blood parameters can have drawbacks (discussed in Section 1.12), they are useful to give a reflection of the animals current status. Especially, when they are used year after year in conjunction with liver and input analysis. Although, the popularity of blood analysis in the current study was far greater than liver; only 12% tested liver samples prior to decision making. Liver copper concentration remains the most accurate reflection of longer-term copper status (discussed in Section 1.12), but appears it is still not widespread in its use.

The findings in this study suggest that farmers typically do not understand copper related problems. The majority of respondents self-evaluated their knowledge (n=120/253) as 'some understanding' with a much higher proportion falling below this level of knowledge than above it (n=92 & 41 respectively). The results supported this lack of understanding. None of the individual farmers were able to correctly identify all the causes of copper related problems and the majority (60%, n=152) identified less than three causes correctly.

This pattern of understanding was also apparent in copper terminology. Out of the 19% that felt familiar with four or more of the key terms only 10% were able to correctly define them. This percentage increased to 15% when partially correct answers were included. Although, no-one defined all the key terms correctly the highest number correct by any individual was six out of seven.

Data was analysed to score respondents by their combined ability to answer the causation and definition questions, this data was compared to their self-assessment of copper-related knowledge (Figure 11). Thresholds for these groups were set by a percentage of correct answers. The thresholds were not set to correspond to a linear sequence; so that modesty and individual differences in the understanding of the categories could be mitigated.

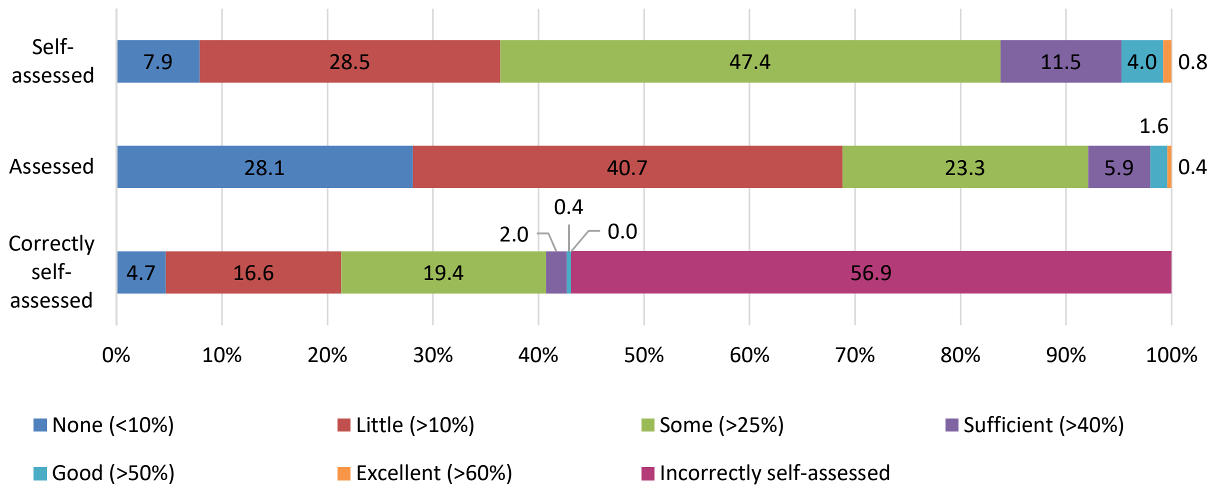

Figure 11: Percentage of total respondents (n=253) able to accurately self-assess their copper-related knowledge in comparison to their self-assessed and assessed data. Brackets denote percentage correct answers considered to reflect knowledge category. Charts labelled with percentage data points for each category.

Overall, knowledge was to be lower than the self-assessment. Respondents who were within the knowledgeable category of 'sufficient' or higher were 8.4% lower than the self-assessment indicated. Less than half (43%) of respondents were able to accurately self-assess their copper-related knowledge. Those with scores of less than 10% (2 questions) correct were considered to have no copper knowledge. Sixty percent of this group correctly assessed their knowledge; which indicates that a large percentage this group had underestimated their copper-related knowledge. Of the respondents who assessed their copper-related knowledge as 'very little' 58% had correctly assessed their knowledge, this group were more likely to be overestimating their knowledge than underestimating it. One respondent in the 'very little' group answered more than 30% correctly, while all the others in this group answered less than 10% correctly. Almost half of the participants felt they had 'some' knowledge. However, only 19% of those who felt they had 'some' knowledge were correct in their self-assessment. Again, respondents were more likely to have overestimated their knowledge as only one respondent exceeded 60% correct. Seventeen percent of those who felt they had 'sufficient' copper-related knowledge were correct in their self-assessment. However, the remaining 81% all overestimated their knowledge. Those who rated their knowledge as 'good' or 'excellent' made up 4.8% of total respondents. However, only 10% of those who rated their knowledge as good answered more than half the questions correctly and none of those who rated their knowledge as excellent answered more than half correctly. This indicates that while half of the farmers conservatively or accurately assessed their own level of understanding, caution should be used when discussing copper related problems with farmers who consider themselves to have a 'good-excellent' understanding of the subject; as it appears these groups are more likely to have overestimated their knowledge than underestimated it.

Several misconceptions were also highlighted in the data. More farmers considered a low bioavailability of copper to be a causal factor in copper problems than insufficient dietary copper. This could suggest a hidden impact from nutritional advisors and supplement sales merchants. Most farmers also appeared unfamiliar with the role of sulphur in the molybdenum and iron antagonist pathways. This is a major misconception, as without sulphur's influence these antagonists do not pose as much risk to copper status. This misconception could lead to misinformed supplemental decisions resulting in the over-supply of copper.

Most farmers were also unfamiliar with the antagonist's mode of action. Few identified the creation of insoluble copper during digestion as a problem. Strangely, farmers were much more familiar with the term 'lock-up'; which perhaps provides an opportunity to enhance and update their knowledge using the terms they are most comfortable with. It also appears the complexity of copper interactions can be misleading. A large proportion of respondents were willing to state that manganese caused copper related problems, and in the free text phosphorus was also stated as a reason for supplementing copper. The complex nature and poor understanding of copper related problems appears to have led to an acceptance that any mineral imbalance will effect copper. It has been suggested by two studies that manganese concentrations of 500 mg/kg DM may exacerbate molybdenum-sulphur and iron-sulphur interactions through competition for the intestinal transporter DMT1 (Legleiter and Spears, 2007; Hansen *et al.*, 2009), and it is possible that some of the respondents in the survey were aware that such an interaction has been suggested resulting in their response. However, since requirements of manganese range from 20-40 mg/kg DM and feedstuff content, while varied, is usually <100 mg/kg DM it is unlikely that this interaction occurs in practice (Suttle, 2010). Additionally, some studies have reported a potential role for a copper-manganese interaction in the development of prion disease (Treiber *et al.*, 2006), it is also possible that some of the farmers who indicated this was a copper interacting element may have misunderstood this information to be a dietary interaction. The lack of understanding across the farming community was also echoed by the inability of farmers to identify the percentage of animals affected by high or low copper within their own animals and across the nation. A lack of understanding in combination with; poorly informed decision making tools, potentially confused and misunderstood information; from vets, advisors and sales representatives, has led to a reliance on basic indicators such as clinical signs and an approach of 'if it ain't broke, don't fix it' in cattle and a 'better not risk it' approach in sheep.

2.6 Conclusion

Farmers have a tendency to supplement their animals on the basis of tradition with general mineral supplements instead of specific ones. Their supplementation routines generally comprise a combination of supplement types which are designed to be easy to administer and last longer; often differentiated at key husbandry events. The approach taken by farmers does not appear to be targeted or routinely revised; despite a large number conducting analysis of input and blood.

In general, copper related problems and their associated terminology are not well understood. Although, the vast majority of farmers are interested in improving their knowledge, a lack of time and the complexity of the subject, in combination with information of varying accuracy, may all be factors which affect this current level of knowledge.

3.0 Copper solubility and interaction modelling I

3.1 Introduction

In the UK, copper supplementation is permitted for all species, including ruminants (discussed in Section 1.4.2. There is an extensive list of permitted copper sources which includes copper from across different mineral groups (European Commission, 2004). In general, copper sources fall into two broad groups; organic and inorganic. The most simple inorganic copper sources are salts; where the copper is ionically bonded to a non-metal, such as; carbon, sulphur, or oxygen. Copper sulphate is historically the most common copper source used in practice. It is considered a cheap, soluble, readily available, and commercially effective source of supplemental copper and it tends to be the standard source used for comparison studies (Kegley and Spears, 1994; Spears, 2003).

More complex inorganic copper sources are also used in practice such as hydroxy minerals; which are covalently bonded. They differ to organic minerals as they are bonded to at least one hydroxyl group; rather than co-ordinately bonded to a carbon-containing ligand (Arthington, 2015). Hydroxy minerals are crystalline making them non-hydroscopic, dust-free and less reactive within feed premixes and blends, providing an increased stability and less lipid oxidation than salts; increasing their popularity in mineral formulations (Miles *et al.*, 1998; Lu *et al.*, 2010; Arthington, 2015).

Organic copper sources include copper amino acid complexes and chelates, which result from combining an amino acid with a soluble copper salt; copper proteinates, where a copper salt is chelated with amino acids and a partly hydrolysed protein; and copper polysaccharide complexes, which involve complexing a copper salt with a polysaccharide solution (Spears, 1996). Chelation involves complexing a ligand which contains a minimum of two functional groups, such as; oxygen, nitrogen, amino or hydroxyl groups, to a copper ion. Each functional group must be capable of donating an electron pair to the copper forming a co-ordinate bond (Spears, 1996). Interest and use of organic copper has increased over the past twenty years. Many (66%) feedlot nutritional consultants from across the USA recommend providing a combination of organic and inorganic sources, with a further 34% recommending using only an organic source. Interestingly, none of the consultants recommended supplementing using only inorganic mineral sources (Samuelson *et al.*, 2016).

3.1.1 Bioavailability

Bioavailability is an important factor in trace mineral provision. In order for a copper source to be considered bioavailable it must possess certain characteristics. It must be able to be absorbed from the gastrointestinal tract and transfer across the mucosa into the enterocytes. It must be able to be transported in the portal blood and taken up by hepatocytes and tissues, and, it must be in a functional form, able to be metabolised and incorporated into the functional units in the body (Crisponi *et al.*, 2010; Suttle, 2010). Throughout this work, the level of bioavailability will refer to the likelihood that copper is present in a form which is able to be absorbed and utilised by the animal. Difficulty in measurement and interpretation, in combination with copper's interactions and disparity in the purity of different supplements make bioavailability difficult to measure accurately. The bioavailability of differing copper sources also ranges considerably; due to the differing chemical properties and characteristics, and can alter in efficacy as a supplement under differing practical conditions (Fry *et al.*, 2013). The bioavailability of copper remains a relatively undefined area which makes comparison of different copper sources difficult. In practice, bioavailability of copper is commonly calculated via a comparison trial *in vivo* and expressed as a percentage value relative to a 'standard' source. Most bioavailability studies use relative rather than absolute values to allow comparison with other studies and findings. Inconsistencies between animal groups, individuals and treatment groups allows relative data to provide a more stable estimation of each source's bioavailability. However, there is no definitive argument for expressing data in this way and there is no standard laboratory test to predict availability of one source relative to another (Cao *et al.*, 2000). Since bioavailability can be measured or predicted through several different assays, and they do not necessarily yield comparable results the researchers choice of analysis can affect the reliability of comparison (Richards, 2010). Despite these difficulties, it is useful to begin to assemble a hierarchy of apparent bioavailability.

3.1.2 Comparing copper sources

When copper sulphate is used as a reference source at 100% bioavailability, the relative bioavailability of copper carbonate is considered 93% (Jongbloed *et al.*, 2002). In comparison, both can maintain blood plasma copper but only copper sulphate increased hepatic concentration (Ward *et al.*, 1996). Perhaps similar to copper carbonate, are copper chloride and copper acetate. Both were found to replete more hepatic copper than copper oxide but less than copper sulphate (Ledoux *et al.*, 1995); forming the hierarchy carbonate > chloride > acetate (Ledoux *et al.*, 1995). Feed-grade copper oxide appears to be essentially unavailable; failing to increase blood copper status markers in copper deficient calves and failing to prevent a decline in copper status where dietary molybdenum and sulphur were high (Kegley and Spears, 1994). Copper lysine has been considered more bioavailable than copper sulphate at 104% using hepatic retention (Jongbloed *et al.*, 2002). Although, it may be similar when measured as plasma copper concentration and caeruloplasmin activity (Ward *et al.*, 1993; Kegley and Spears, 1994). Under stressful conditions, the retention of copper appeared five times higher from copper lysine than copper sulphate (Nockels *et al.*, 1993; Spears, 1996). Tribasic copper chloride (TBCC) may also have a higher bioavailability than copper sulphate, between 121-196% in animals which also had high sulphur and molybdenum intakes (Spears, 2003; Spears *et al.*, 2004), and recent work using liver copper concentration to calculate relative bioavailability in steers fed a high molybdenum and sulphur diet found relative bioavailabilities of 112 and 100% for TBCC and copper sulphate respectively (Vanvalin *et al.*, 2019). Although, copper sulphate and TBCC appear to have similar bioavailability in diets which are low in molybdenum (Spears *et al.*, 2004; Arthington and Spears, 2007; Faulkner *et al.*, 2017). In low molybdenum diets, copper proteinate and copper sulphate were similar in bioavailability. When molybdenum was increased, copper proteinate appeared to have higher bioavailability; resisting decreases in liver and plasma copper concentration (Kincaid *et al.*, 1986; Ward *et al.*, 1996). Although, others have reported no difference in similar trials (Wittenberg *et al.*, 1990a). Copper glycinate may have greater bioavailability than copper sulphate by 150%; based on liver copper status when animals were fed a diet high in molybdenum and sulphur (Hansen *et al.*, 2008). Methionine hydroxy copper has also reduced egestion of copper in faeces, representing more effective utilisation, alongside increasing blood plasma and liver copper concentrations in comparison to copper sulphate (Pal *et al.*, 2010; Wang *et al.*, 2012).

3.1.3 Compound chemistry and bioavailability

The differences in behaviour of the copper sources can be potentially explained through differences in the chemical properties of the copper compounds. The ionic soluble sources such as copper sulphate and chloride are expected to be rumen soluble and remain soluble through digestion (Ward *et al.*, 1996). However, these labile; easily broken down or displaced, sources are expected to interact readily with antagonists. Ionic sources such as copper carbonate are expected to have a low solubility in the rumen. Copper carbonate has a low solubility in water of <1% which notably increases (74%) after prolonged incubation with hydrochloric acid (Ward *et al.*, 1996), suggesting it may avoid lability in the rumen, but become soluble after abomasal digestion. The copper sources which behave in this manner are referred to as 'rumen by-pass' sources. In contrast, copper oxide remains insoluble throughout digestion explaining the absence of responses in feed-grade products. However, copper oxide in a bolus may provide a more effective utilization of this copper source; able to increase hepatic copper (Langlands *et al.*, 1989; Yost *et al.*, 2002; Balemi *et al.*, 2010). The discrepancy between administration method and availability may be attributed to the faster transit time of powder in comparison to copper oxide needles. Powder may pass through the acidic conditions of the abomasum before becoming soluble (Kegley and Spears, 1994; Spears, 2003). Whereas the needles are retained, embedded in the gastrointestinal wall, releasing copper over a period of weeks to months (Cameron *et al.*, 1989; Langlands *et al.*, 1989; Suttle, 2010). In comparison to copper sulphate and an organic copper complex, a copper oxide bolus produced a higher caeruloplasmin concentration and faster liver copper accumulation (Yost *et al.*, 2002). Although, liver accumulation in a copper deficient diet found a copper oxide bolus to be only 77% as effective as a copper sulphate drench (Balemi *et al.*, 2010).

Hydroxy copper sources such as TBCC have low rumen solubility. The observed higher bioavailability has been theorised to stem from a similar mode of action to copper carbonate and it also considered to 'by-pass' the rumen (Miles *et al.*, 1998; Spears, 2003, 2013; Arthington, 2015). However, in these complexes the copper is covalently bonded rather than ionically bonded. The covalent bond is considered strong enough to withstand interactions, but weak enough to pass the copper to the biological chaperones allowing its absorption (Arthington, 2015).

Organic sources may have similar solubility to soluble ionic sources in the rumen. Copper acetate, lysine, chloride and sulphate have been reported to have similar solubility in both *in vivo* and *in vitro* studies (Charmley and Ivan, 1989; Ward and Spears, 1993; Kegley and Spears, 1994; Luo *et al.*, 1996). Further *in vitro* studies using different solvents have also concluded similar findings for these copper sources (Luo *et al.*, 1996). However, it is theorised that organic copper sources should be more bioavailable than the inorganic forms; as the co-ordinate bonds formed between the metal and ligand should protect the copper from the interactions encountered in the rumen (Hansen *et al.*, 2008; Spears, 2013). However currently, there is no conclusive evidence to suggest that organic sources are more bioavailable than their inorganic counterparts. Various studies have concluded opposing points or no difference (Wittenberg *et al.*, 1990a; Ward and Spears, 1993; Ward *et al.*, 1993; Pott *et al.*, 1994; Rabiansky *et al.*, 1999; Balemi *et al.*, 2010; Karkoodi *et al.*, 2012; Lippolis *et al.*, 2017). Additionally, concern that copper complexed to amino acids may be absorbed as an amino acid rather than a trace mineral; negating the homeostatic control of copper absorption have been raised (Bidewell *et al.*, 2012). In trials, copper-amino acid complexes were absorbed with great efficiency in comparison to inorganic copper salts, including where levels of sulphur and molybdenum are high (Hansen *et al.*, 2008). This may be problematic, as copper could continue to absorb in animals of high copper status due to a lack of homeostatic regulation; potentially increasing copper accumulation and the potential for haemolytic crisis (Bidewell *et al.*, 2012). However, Turner *et al.*, (1987) reported no difference in the rate or manner of absorption of copper from copper histidine, copper glutamine, copper lysine or copper sulphate in everted sheep intestine suggesting this may not be a practical concern.

Organic copper sources represent copper bound to a range of carbon containing ligands (Vieira, 2008). Only polydents; where the copper atom is bound by more than one bond, are able to form chelates (Vieira, 2008). Most organic chelates used in nutrition are tetradentate ligands. Vohra and Kratzer, (1963) demonstrated that chelates with high stability constants protect copper from interactions in the digestive tract (Ashmead, 1993). The copper component is held in an inert form due to co-ordinate bonds formed by amino ligands (Vieira, 2008). Co-ordinate bonds are a type of covalent bond, but differ in the way electrons are shared. Covalent bonds share the electrons from both atoms, while co-ordinate bonds are contributed from one atom to the other (Hill and Holman, 2011). Co-ordinate bonds form a stable molecule less susceptible to interactions and are protected from acidic dissociation in the abomasum (Ashmead, 1993). When chelated, minerals reach the small intestine they are able to be absorbed intact through the mucosa of the jejunum and into the blood plasma (Acda and Chae, 2002). However, if a chelate does not remain intact through digestion it has no advantage over soluble salts (Kratzer and Vohra, 1986; Ashmead, 2001). Metals which are bound to organic ligands by co-ordinate bonds are able to dissociate during metabolism, whereas covalent bonds cannot (Vieira, 2008). The co-ordinate bond aligns the metal and ligand in such a way that the electrons available from the donor atoms are positioned close to those from the metal (Vieira, 2008). The type of donor atom as well as the denticity of the ligand influences the complex formation and its stability (Helsel and Franz, 2015). As the denticity of the ligand increases the stability of the complex also increases (Helsel and Franz, 2015). The stability constant should be high enough to allow the chelate to cross the intestinal cell membrane into the cytoplasm and yet be low enough that the cytoplasmic ligands, such as glutathione, are capable of removing the metal from the absorbed amino acid chelate by complexing to the absorbed metal and releasing the metal ions for assimilation into the animals system (Kratzer and Vohra, 1986; Ashmead, 2001; Acda and Chae, 2002). If the bond formed between the metal and the chelate is too strong to be broken down by metabolism the entire chelate, including its mineral component, will be lost through egestion.

The behaviour of chelated minerals makes the study of synthetic chelates interesting. Ethylene-diamine-tetra-acetic acid (EDTA) forms a hexadentate ligand forming 6 co-ordinate bonds to the copper atom. Although it is not on the approved feeds list copper EDTA is suggested to be 95% bioavailable (Ammerman *et al.*, 1995), and to have a similar absorption value to copper sulphate in sheep (Suttle and Price, 1976). Most studies involving copper EDTA have used parenteral, sub-cutaneous dosing rather than feed trials. In these studies, copper EDTA was able to prevent the onset of copper deficiency and increased liver copper concentration (Allen and Mallinson, 1984; Langlands *et al.*, 1986; Suttle, 2010; Fazzio *et al.*, 2017). However, interest in copper EDTA subsided as it was thought the stability constant was sufficiently high to prevent it being an effective mineral source (Ding *et al.*, 2011).

3.1.4 Determining bioavailability

The primary step determining the bioavailability is solubility (Ibrahim *et al.*, 1990). Solubility allows transfer across the mucosa and into the animal's system (Acda and Chae, 2002; Suttle, 2010). A mineral which remains insoluble throughout digestion fails the criteria outlined in Section 3.1.1 and remains unavailable through digestion only to be egested in the faeces (Crisponi *et al.*, 2010).

The antagonist pathways which inhibit copper absorption have two dominant modes of action. Primarily, within the digestive tract they are able to form complexes with other dietary components which render them insoluble or unavailable for absorption (see Sections 1.7 & 1.8). Secondly, copper can compete for transporters in absorptive regions (see Section 1.10). It is this first mode of action which is attributed to the two most biologically relevant antagonisms; copper-molybdenum-sulphur, and copper-iron-sulphur, which are both posited to act upon soluble copper in the rumen rendering it insoluble.

The bioavailability studies discussed in Section 3.1.2 have made their conclusions for bioavailability based on changes in either liver status or blood parameters, or both, and have hypothesised the likely digestive activity of the copper compounds *in vivo* based on their chemical properties rather than through direct testing.

It has yet to be established if the antagonists act through a purely chemical means or if they rely on an undetermined factor within the complexities of the rumen environment. Since the solubility of copper is paramount to its bioavailability. The logical starting point in elucidating the mode of action for these antagonists is to investigate the chemical nature of these interactions with copper sources *in vitro*. Initially, exploring direct chemical interactions using purified, deionised water as a substrate under simplistic rumen conditions including; temperature, retention time, pH and anoxia.

3.2 Aims & Objectives

The aim of this work was to establish the effects of sulphur and iron on copper solubility from a range of copper sources without the complications of a biological matrix. The baseline solubility of different copper sources was determined and used to compare the effects of sulphate, sulphide and a mixture of both, with and without iron, in deionised water at ruminal pH and incubation time under anoxic conditions.

3.3 Materials & Methods

3.3.1 Replicated rumen parameters

In this work, the rumen environment was replicated through buffering samples to pH 6.2 in line with grazing ruminal pH. Retention time of a grazing ruminant was replicated through incubation for 25 h at 39 ˚C with gentle agitation to simulate rumen contraction and mixing (as discussed in Section 1.5). Concentrations of minerals were calculated to represent biological parameters relevant to both cattle and sheep. Typical rumen liquid volume (not total rumen volume), DMI and mineral concentration (as DM), were required to calculate molar mineral concentrations representative of the rumen.

For cattle, typical rumen liquid volume may vary between 50-80 l (Moloney *et al.*, 1993; Brown *et al.*, 2008; Richter, 2011). In this trial, an average fluid volume of 70 l (Moloney *et al.*, 1993) was selected alongside a DMI of 14 kg/day which falls within the wide range of 11-30 kg/day reported across different cattle production systems (NRC, 2000, 2001). In sheep, rumen liquid volume ranges from 2.5-9 l (Ulyatt, 1964; Purser and Moir, 1966; Rémond *et al.*, 1996) an average of 6 l was selected for sheep alongside a DMI of 2 kg/day (range 1-5 kg/day) (NRC, 2007). NRC (2001, 2000, 2007) guideline requirements were used for copper, iron and sulphur. Sulphide concentration was calculated ~50% of maximum and in line with previously reported concentrations of 6 mg/l (Anderson, 1956; Bray, 1963; Gawthorne and Nader, 1976; Laurie, 2000). Inclusion rates and molar concentrations are shown in Table 2.

Table 2: Inclusion concentrations within NRC guidelines for cattle and sheep and final molar concentrations used to replicate rumen mineral concentrations.

Mineral	Cattle	Sheep	Molarity
Copper	11 mg/kg DM	6 mg/kg DM	0.03 mM
Iron	50 mg/kg DM	40 mg/kg DM	0.2 mM
Sulphur	3 g/kg DM	2 g/kg DM	0.02 M

3.3.2 Solubility benchmarking

To create a benchmark for solubility without antagonist effects for comparison, each of the copper sources listed in Table 3, alongside iron chloride ($FeCl_3{\cdot}6H_2O$, Fisher Scientific, Loughborough, UK. >99%) were prepared into 0.1 M solutions in purified, deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm). Copper solutions were further diluted to create 4 mM solutions of each copper source. Iron was further diluted to a 24 mM solution.

Table 3: Copper, iron and molybdenum sources used for solubility trials with manufacturer information and purity.

Mineral source	Manufacturer	Purity
Copper sulphate ($CuSO_4{\cdot}5H_2O$)	Acros Organics, New Jersey, USA	99-102%
Copper chloride ($Cu^{2+}Cl_2{\cdot}2H_2O$)	VWR International Ltd, Lutterworth, UK	≥99.0%
Copper oxide (CuO)	Fisher Scientific, Loughborough, UK	>99%
Copper carbonate ($CuCO_3{\cdot}Cu(OH)_2$)	Fisher Scientific, Loughborough, UK	>99%
Tribasic copper chloride ($Cu_2(OH)_3Cl$)	Intellibond®, Selko®, Tilburg, NL	≥97.0%
Copper EDTA ($Cu(OOCCH_2)_2NCH_2CH_2N(CH_2COONa)_2$)	Sigma-Aldrich, Dorset, UK	≥97.0%
Copper proteinate	Bioplex® Alltech® USA	10% elemental copper
Copper acetate ($Cu(CH_3COO)_2$)	Sigma Aldrich, Dorset, UK	≥98.0%
Copper$^+$ chloride (Cu^+Cl)	Fisher Scientific, Loughborough, UK	>99%

Copper solution (25 µl) was pipetted into polypropylene tubes (Sarstedt Ltd., Leicester, UK) containing 3.5 ml of deionised water (n=4-6). Solutions were made up to a total volume of 4 ml and pH adjusted to 6.2 using (475 µl) citrate buffer prepared from tri-sodium citrate dihydrate (VWR International Ltd, Lutterworth, UK. >99.0%) and citric acid monohydrate (Sigma Aldrich, Dorset, UK. >99.0%) in a 56:1 ratio, calibrated using a pH meter (Orion star 3, Thermo Fisher Scientific, Waltham, USA).

Solutions were incubated in a shaking water bath (OLS200, Grant instruments, Royston, UK) set at 39°C, 30 rpm for 25 h prior to sampling.

After incubation, solutions were centrifuged at 2,000 *g* for 10 minutes (Allegra X-22, Beckman Coulter, High Wycombe, UK) and supernatant analysed using inductively coupled plasma mass spectrometry (ICP-MS).

3.3.2.1 Multi-elemental analysis using ICP-MS

Multi-element analysis of solutions was undertaken by ICP-MS (XSeriesII, Thermo Fisher Scientific, Waltham, USA). Samples and calibration standards were diluted (500 µl sample with 9.5 ml diluent) in a diluent containing 0.1% of a non-ionic surfactant ('Triton X-100' and 'antifoam-B'; Sigma Aldrich, Dorset, UK), 2% methanol and 1% HNO_3 (Fisher Scientific, Loughborough, UK. >99%) including the internal standards Ir (5 mg/l), Rh (10 mg/l), Ge (50 mg/l) and Sc (50 mg/l). All calibrations were in the range 0-50 mg/l (Claritas-PPT grade CLMS-2 from Certiprep/Fisher Scientific, Loughborough, UK).

ICP-MS was conducted in 'collision-cell technology with kinetic energy discrimination' (CCT-KED) mode using 7% H_2 in He as the hexapole collision cell gas; reducing polyatomic interference with aspiration though a single sample line and glass concentric nebuliser (Thermo Fisher Scientific, Waltham, USA; 1 ml/min). Results were then calibrated to pre-diluted concentrations and adjusted for background using blank correction.

3.3.3 Effects of sulphur and iron on copper solubility

All reagents were prepared using deionised water (Purite hp 160 , Suez, UK. 17 MΩ cm) as a substrate using the method described in Section 3.3.2. Sulphur from sodium sulphate (Na_2SO_4, VWR International Ltd, Lutterworth, UK. >99%) and sodium sulphide ($Na_2S{\cdot}9H_2O$, Fisher Scientific, Loughborough, UK. >98%) were prepared into 2.4 M and 0.24 M solutions respectively using purified, deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm). Sulphide was prepared and used within a microaerophilic hood (MACS VA500, Don Whitley Scientific, Bingley, UK. <0.5% O_2) to inhibit oxidation. Where the experimental design indicated, 25 µl of iron solution, followed by 25 µl of sulphate, sulphide or both (in the order sulphate then sulphide) solutions in their respective concentrations, were added to each of the copper sources in buffer solution (450-425 µl as indicated) creating a solution with a total volume of 4 ml. Where sulphide or reduced copper (Cu^+Chloride) was used, anoxia was maintained using a microaerophilic hood (MACS VA500, Don Whitley Scientific, Bingley, UK) whenever samples were uncapped. Each copper source was tested in a randomised block design using one-way ANOVA comprising the treatments; 'control', 'sulphate', 'sulphide', 'sulphate & sulphide' (sulphur mix) and 'iron with sulphate & sulphide' (iron with sulphur mix). Controls containing only buffer solution were prepared for each copper source alongside blanks containing only deionised water and buffer.

3.3.4 Analysis

Samples were analysed through ICP-MS for total elemental concentration of copper, iron, sulphur and molybdenum according to the protocol described in Section 3.3.2.1. The data was then adjusted to account for background readings from the substrate controls and data transformed to give a percentage soluble copper in comparison to initial concentration.

3.3.5 Data analysis

The data was analysed for descriptive statistics and statistical significance using general linear model ANOVA with Tukey comparisons using Minitab v.18 (Minitab, Pennsylvania, USA). In all relevant tests an α value of $P<0.05$ was used as the cut-off for significance. Subsequent increased levels of significance were determined at $P<0.01$, $P<0.001$ and $P<0.0001$. Results are expressed ± standard deviation.

3.4 Results

3.4.1 Baseline solubility

The copper compounds soluble in water were the ionic salts; copper chloride and copper sulphate, the chelated compounds; copper proteinate and copper EDTA, and the most soluble source was the amino acid chelate; copper proteinate. Solubility values for these copper sources ranged from 92-112% (±24) soluble. Less ($P<0.0001$) soluble compounds ranged from 62-12% (±19) soluble. At the upper end of this range was copper acetate at 62% (±7) soluble, followed by copper carbonate and copper oxide (32±32% and 26±16% respectively). The most insoluble compounds were Cu^{+}chloride and TBCC (17±21% and 12±1% respectively).

3.4.2 Effect of sulphur source on copper solubility

The mean solubility value across all copper compounds without sulphur was 64% (±46), this was not significantly different when sulphate was introduced (55±34%). Where sulphate was added, soluble copper sources remained highly soluble ranging from 61-101% (±19) soluble. Copper acetate remained similar at 56% (±0.7) soluble and the insoluble compounds continued to give the lowest solubility. Figure 12 shows the relative difference in soluble copper reported for each copper source alongside sulphur treatments.

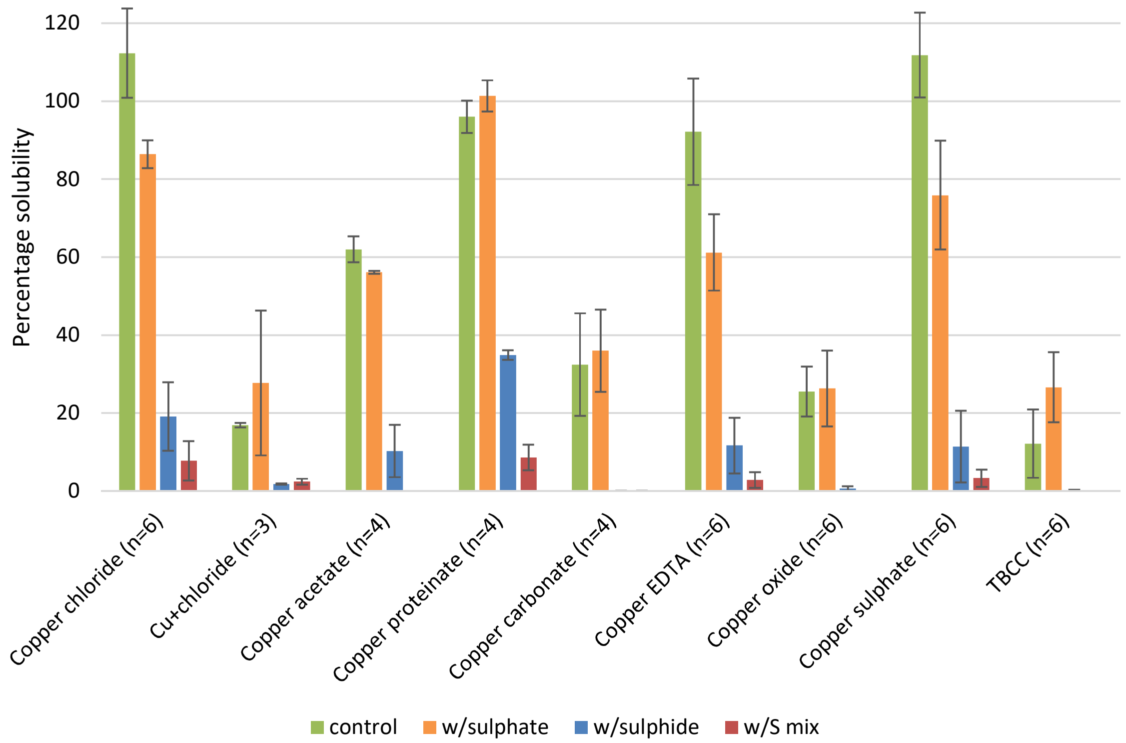

Figure 12: Percentage soluble copper for each source in comparison with baseline solubility (left bar) compared to sulphur sources. Error bars denote standard error of the mean.

Where sulphide was added mean solubility across all copper sources ($P<0.0001$) reduced to 9% (±16). Copper proteinate had the highest solubility (35±2%) which was not significantly different to the other soluble sources (range 10-19±2-23% soluble). Interestingly, the results also reported that these copper sources were not significantly different to Cu^{+}chloride despite its mean solubility value being 2±0.3%. This may be due to a lower number of replicates (n=3) of this compound reducing the reliability of the data and a higher standard deviation in the comparing compounds. The insoluble copper sources also appeared to have been effected by the presence of sulphide, despite having a lower initial solubility benchmark solubility values for these copper sources were all reduced to <1±0%.

The sulphur mix also ($P<0.0001$) reduced mean solubility in comparison to sulphate and non-sulphur benchmark, despite being lower (2.7±6%) this was not significantly different to sulphide. Under the influence of both sulphate and sulphide, copper proteinate had the highest solubility (27±7%). The other copper sources were all (P<0.05) lower and had reduced to <10±7% which was not significantly different to each other.

3.4.3 Effect of iron on copper solubility

Where iron was added alongside sulphate and sulphide the results were not significantly different to their respective treatment without iron for all copper sources except copper proteinate (Figure 13). Copper proteinate had the highest soluble copper (27±9%). Interestingly, this solubility was (P<0.05) higher than its respective sulphur mix value without iron and was (P<0.0001) higher than all the other copper sources. The mean solubility value across all compounds was 5±3%. In addition to copper proteinate, only copper chloride and copper sulphate returned a soluble copper value in this test (9±12% and 0.9±0.7% respectively). Although, this was not significantly different to the insoluble copper sources which were all 0±0%.

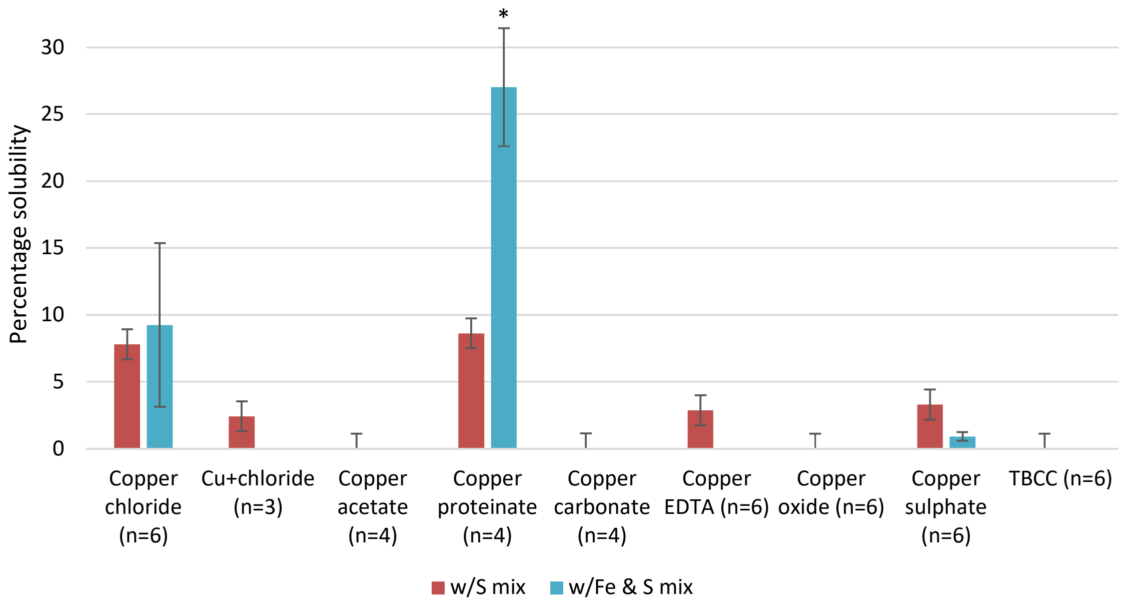

Figure 13: Percentage soluble copper for each source with sulphur mix vs sulphur mix with iron. Error bars denote standard error of the mean. Asterisk denotes significant difference (P<0.05).

To try and distinguish if iron had an effect on copper solubility; copper chloride, Cu^{+}chloride and copper sulphate were combined with iron in each of the previous treatments. There was no significant difference between the presence or absence of iron on the mean solubility of the compounds across the tests. However, the effect of sulphur source was still present ($P<0.0001$) and both copper chloride and copper sulphate were ($P<0.0001$) more soluble than Cu^{+}chloride across the tests.

No significant difference was found in iron solubility across the different treatments or in comparison from baseline solubility to where copper was present (Figure 14).

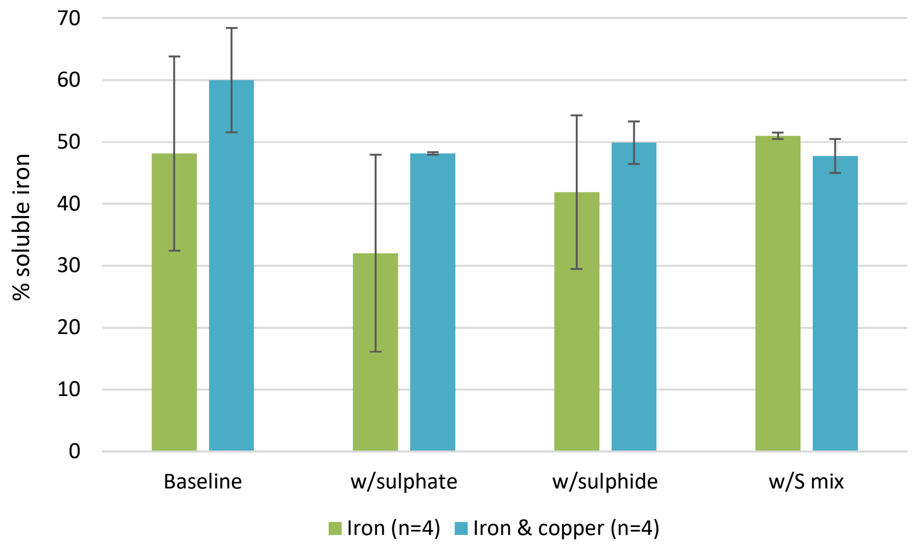

Figure 14: Percentage soluble iron with and without copper chloride. Error bars denote standard error of the mean.

3.5 Discussion

The solubility benchmark findings were as expected from textbook values and are in agreement with studies which have measured the solubility of these compounds in the rumen or in rumen-replicated *in vitro* trials (discussed in Section 3.1.3). Copper sulphate, copper chloride, copper proteinate and copper EDTA were all found to be highly water soluble; which is concurrent with evidence from practice showing these compounds are considered readily absorbable and highly bioavailable (Charmley and Ivan, 1989; Kegley and Spears, 1994; Ledoux *et al.*, 1995; Jongbloed *et al.*, 2002; Spears, 2003). However, their solubility all appeared to be heavily affected by the introduction of sulphide; corresponding with previous *in vitro* and *in vivo* work which has demonstrated an effect of copper sulphide formation (Mills, 1960; Bird, 1970; Suttle, 1974b; Dick *et al.*, 1975; Ivan, 1988; Grace *et al.*, 1997; Spears, 2003). As discussed in Section 1.9 around a third of dietary sulphur can be converted by the rumen bacteria into sulphide (Lewis, 1954; Gawthorne and Nader, 1976), which then reacts with copper to form insoluble copper sulphide as $Cu^{+}S$ (Mountain and Seward, 1999). The present model could not replicate the dynamics of the rumen in terms of bacterial sulphate conversion but did show a clear effect of copper sulphide formation.

In line with previous work; copper carbonate, TBCC and copper oxide were all found to be relatively insoluble in water (Kegley and Spears, 1994; Ward *et al.*, 1996; Spears, 2003). These sources also appeared to be affected by sulphide, the lesser extent to which they were affected is likely linked to the lower initial solubility of these compounds. This current work is in agreement with the theory discussed in Section 3.1.3 that they may act as 'rumen by-pass' compounds (Miles *et al.*, 1998; Spears, 2003, 2013; Arthington, 2015). Due to the systemic implications of thiomolybdate absorption (discussed in Section 1.7), it could be considered that the use of rumen 'by-pass' copper products alone is ultimately not beneficial to the animal. Their use could potentially lead to the absorption of un-bound thiomolybdate through the rumen, due to the absence of labile copper at the major site of thiomolybdate formation (Kelleher *et al.*, 1983; Mackenzie *et al.*, 1997a). The presence of systemic thiomolybdate may subsequently interfere with cuproenzymes and proteins giving rise to clinical signs. The absorption of thiomolybdate from the rumen may only leave a small amount of thiomolybdate to bind the, now available copper, once it reaches the small intestine. Thus, allowing normal copper absorption in the small intestine, which could subsequently accumulate in the liver to elevated concentrations, increasing the risk of haemolytic crisis (Kendall and Bone, 2013).

In the present work, copper proteinate had the highest solubility across the sulphur challenges. These results correspond with previous *in vivo* work where copper proteinate appeared highly available (Kincaid *et al.*, 1986; Wittenberg *et al.*, 1990a; Ward *et al.*, 1996). This finding also supports the work of Ward *et al*., (1996) who found copper proteinate depleted less copper from the liver than sulphate or carbonate, but contrasts to other trials where its performance did not differ to ionic sources (Wittenberg *et al.*, 1990a). The results of the present work indicated copper proteinate increased in solubility in the presence of iron (Figure 13). No current literature exists to determine if this effect has been observed in practice. Suggesting further investigation could be required to determine if this result is an artefact of experimental error or reflects a difference in behaviour for this copper source.

Copper acetate performed in a similar manner to the insoluble sources in this trial. Previous *in vivo* trials showed a bioavailability similar to copper chloride (Charmley and Ivan, 1989; Ledoux *et al.*, 1995), however, this did not appear to be the case in the present work.

Although not approved for animal use, monovalent copper chloride (Cu^+chloride) was also investigated to assess the impact of oxidation state on the interaction with sulphur and iron. Cu^+chloride was the only monovalent (Cu^+) source used in this work. As expected, this source was also insoluble and performed in a similar manner to the other insoluble sources in each test. Previous theory suggests that Cu^+ would be the preferred substrate for sulphide over the Cu^{2+} sources (Mountain and Seward, 1999). However, the clear reduction in solubility of the soluble sources where sulphide was present suggest that Cu^{2+} is also a suitable substrate for this interaction.

Where iron was introduced there was no evidence of an insoluble compound forming. There was no reduction in solubility of either copper or iron in comparison to their solubility where only one of the metals was introduced (Figure 15). This lack of effect was irrespective of the copper, iron or sulphur source and was also not significant for Cu^+chloride suggesting that oxidation state does not elicit this reaction.

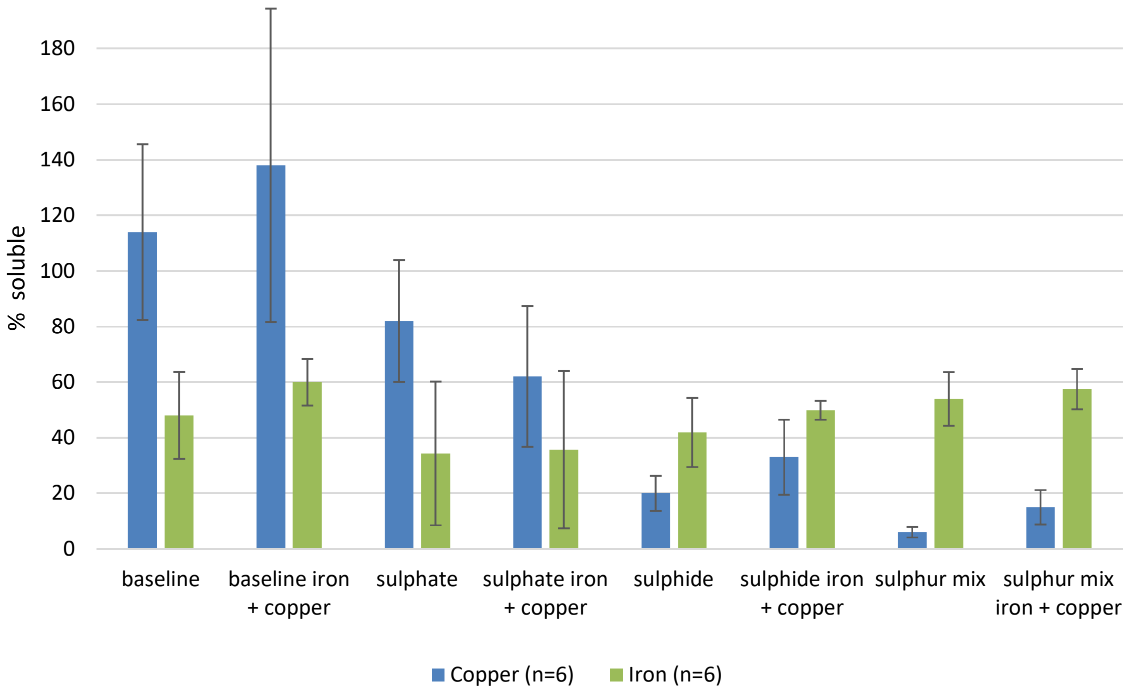

Figure 15: Percentage soluble copper and iron from copper chloride and copper sulphate combined. Data shows solubility for each metal individually and combined for each sulphur solution. Error bars denote standard error of the mean.

The absence of any effect of iron indicates that this interaction is not a simple chemical interaction. Replication of the rumen environment in terms of pH, temperature, incubation and anoxia were not sufficient to elicit this interaction *in vitro*. However, it was interesting to note that copper proteinate, and to a lesser extent copper chloride, appeared to increase in solubility where iron was included without having an effect on iron solubility. Further investigation of this effect is warranted to ascertain if this resulted from experimental error or if it is an influence of relevance *in vivo.*

3.6 Conclusion

Copper solubility across the copper sources without sulphur present was in line with expected textbook values. The results of this trial clearly demonstrated sulphide, irrespective of the presence of sulphate, had the greatest effect on copper solubility. This effect was present across all the copper sources but was most pronounced on the soluble sources. The reduction in solubility where sulphide is present can be attributed to the formation and subsequent precipitation of copper sulphide which is known to be almost completely insoluble and unabsorbed (Huisingh *et al.*, 1974; Ward, 1978). The reduction in copper solubility from sulphide was more notable than expected. This interaction is commonly overlooked in practice and appears able to significantly reduce the solubility of copper compounds. Although it is likely that the antagonist pathways involving molybdenum-sulphur and iron-sulphur act at a higher affinity *in vivo* and are more biologically relevant, it is worth considering the contribution of sulphur alone to copper availability.

The present results suggest that simplistic rumen parameters replicated using de-ionised water used was insufficient to elicit the formation of the iron-copper-sulphur compound. The next logical step in elucidating this mode of action is to continue these studies using rumen fluid as a substrate.

4.0 Copper solubility and interaction modelling II

4.1 Introduction

Solubility trials in deionised water demonstrated solubility patterns which shared some similarity to those found in bioavailability studies (discussed in Section 3.5). However, the absence of insoluble compound formation where copper, iron and sulphur were present under rumen simulation of; temperature, incubation, rhythmic contraction, pH and anoxia demonstrates that this antagonist requires further replication of the rumen environment in order to elicit its formation. The complexities of the biological matrix as discussed in Section 1.5 combined with the widespread observations of this antagonist's effects (discussed in Section 1.8) suggest that the interaction between these dietary elements is more complex than a simple chemical reaction. In order to further study this interaction a more accurate replication of the rumen environment is required. A second solubility trial to test the solubility of copper compounds and the mode of action of both iron-sulphur and molybdenum-sulphur as copper antagonists was conducted using the previously defined rumen simulated parameters. These second trials utilised warmed and strained rumen fluid as a substrate. The presence of microflora and solid-liquid phase particulate are complex and potentially influential aspects of the rumen environment on the solubility of copper.

4.2 Aims & Objectives

The aim of this work was to establish the effects of sulphur, iron and molybdenum on baseline copper solubility in rumen fluid under rumen simulated conditions and to determine any changes which occur under abomasal simulated conditions.

4.3 Materials & Methods

4.3.1 Replicated rumen parameters

The parameters used in this work were consistent with those used in Section 3.3.1, to allow for comparison across the substrates to be made. In addition to copper, iron and sulphur concentrations a typical elevated pasture molybdenum of 2-3 mg/kg DM based on the previously described rumen volume and DMI was used to provide the molar concentration for molybdenum. In addition to ruminal replication, an abomasal phase was also added to this work as an acid incubation for 1 h at a concentration of 0.01 M HCl consistent with the biological secretion of hydrochloric acid and retention time in the abomasum (discussed in Section 1.5).

4.3.2 Solubility benchmarking

Rumen-simulated solubility of the copper sources (listed in Section 3.3.2 Table 3, with the omission of Cu^{+}chloride) were benchmarked to provide a solubility value without antagonists; using the procedure outlined in Section 3.3.2. Copper sources were added to buffered (pH 6.2), warmed and strained rumen fluid without the addition of any other elements and incubated as previously described.

An abomasal-simulation step was added to this trial. Incubated samples were filtered through an 11 µm pore size filter paper (№1, Whatman, Maidstone, UK). Once drips had ceased, filter papers were inverted and soaked in 10 ml 0.01 M HCl for 1 h. Filtrate was centrifuged at 2,000 *g* for 10 minutes (Allegra X-22, Beckman Coulter, High Wycombe, UK) and analysed using ICP-MS (described in Section 3.3.2.1). After incubation, filter papers were removed and samples centrifuged using the previously described settings and analysed using ICP-MS.

4.3.3 Effects of sulphur and iron on copper solubility

All reagents were prepared and added to warmed and strained rumen fluid as a substrate using the method described in Section 3.3.3. Rumen fluid was obtained using the rumen sampling device shown in Figure 21, from the 4 fistulated adult female Holstein-Friesian cattle used in the later trials (Chapter 5 & 8). Approximately 1l of rumen fluid was collected from each animal while they were not on any experimental trial. The fluid was subsequently pooled to provide the substrate for this study. Each copper source was tested in a randomised block design using one-way ANOVA comprising the treatments; 'control', 'sulphate', 'sulphide', 'sulphate & sulphide' (sulphur mix) and 'iron with sulphate & sulphide' (iron with sulphur mix). Controls containing only buffer solution were prepared for each copper source alongside blanks containing only substrate and buffer. After incubation, samples were tested for rumen-simulated soluble and abomasal-simulated soluble fractions using the methods previously described.

4.3.4 Interactions between copper, sulphur, iron and molybdenum

Reagents were prepared using the protocol described in Section 3.3.3 into warmed and strained rumen fluid. Copper chloride was used as the copper source for these tests. Where the experimental design indicated, 25 µl of iron solution, followed by 25 µl of molybdate solution and then sulphate, sulphide or both solutions in their respective concentrations, were added to each of the copper sources in buffered rumen fluid creating a solution with a total volume of 4 ml. Where required, anoxia was maintained using a microaerophilic hood (MACS VA500, Don Whitley Scientific, Bingley, UK) whenever samples were uncapped.

Mineral combinations tested were; Mo, Cu+Mo, Fe+Mo and Cu+Fe+Mo. Using sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$, Fisher Scientific, Loughborough, UK. >99%) diluted to 0.8 mM.

To each of these combinations sulphur sources were added in a one-way ANOVA as one of; 'none', 'sulphate', 'sulphide' and 'sulphate & sulphide' (sulphur mix). Samples were tested for rumen-simulated soluble and abomasal-simulated soluble fractions using the methods previously described.

4.3.5 Analysis

Samples were analysed for total elemental concentration through ICP-MS according to the protocol described in Section 3.3.2.1.

4.3.6 Data analysis

Data was adjusted and analysed using the procedure described in Section 3.3.5 and the previously determined significance values.

4.4 Results

4.4.1 Baseline solubility

The mean baseline solubility in rumen fluid across all the copper compounds was 33% (±18) which was ($P<0.0001$) lower than the respective value in water (Figure 16). The results indicated copper sulphate, copper chloride, copper acetate, copper EDTA and copper proteinate were the most soluble in rumen fluid. Soluble copper values for these compounds ranged from 33-49% (±3-14%) but were not significantly different to each other. Copper acetate was the least soluble of these compounds and was not different to copper carbonate. Copper carbonate alongside the other water insoluble sources had the lowest solubility values ranging from 8-19% (±0.9-9%).

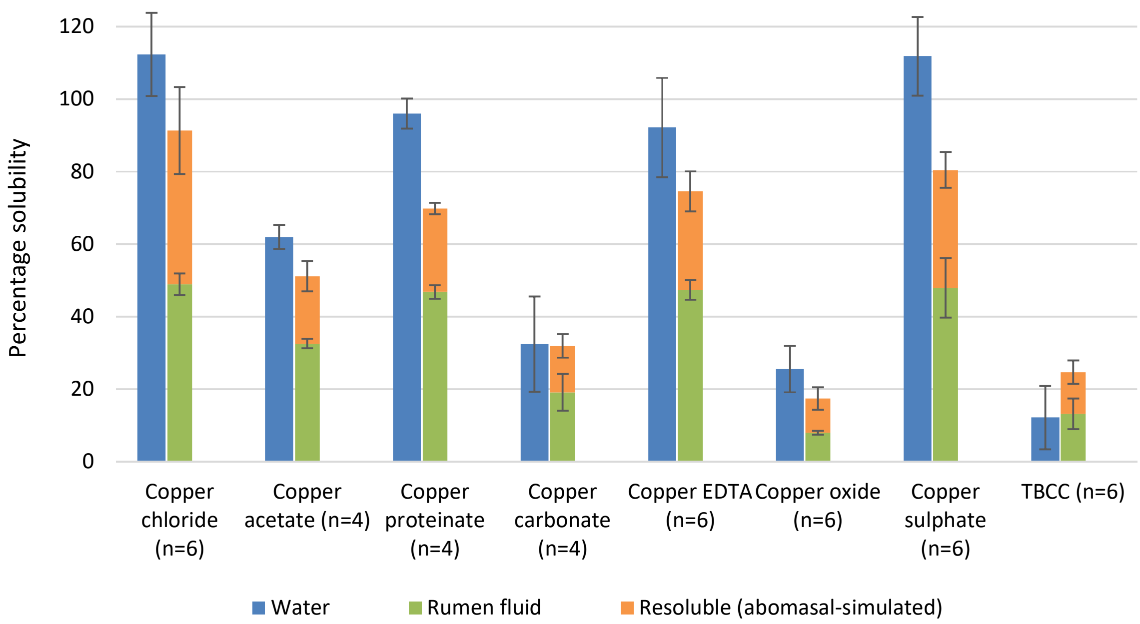

Figure 16: Percentage soluble copper for each source in de-ionised water (data reported in Chapter 3) and rumen fluid for rumen-simulated solubility and abomasal-simulated solubility (resoluble). Error bars denote standard error of the mean.

There was a ($P<0.01$) difference between the resoluble copper obtained after abomasal-simulation from the different sources. The most resoluble copper was obtained from copper chloride. An additional 42% (±24) was able to be solubilised after abomasal simulation.

Bringing the total soluble percentage for this source up to 91%. Copper sulphate, copper EDTA, copper proteinate, and copper acetate also had resoluble copper (33±10, 27±11, 23±3, 19±8% respectively). Copper carbonate, tribasic copper chloride and copper oxide produced the least resoluble copper (13±7, 12±6, 9±6% respectively) which was ($P<0.05$) less than copper chloride.

4.4.2 Effect of sulphur source on copper solubility

The mean solubility across all copper compounds did not differ in the presence of sulphate to rumen fluid without any sulphur. Unlike baseline, there was no difference between substrates where sulphate was introduced. The solubility across all compounds in rumen fluid was 30% (±7) compared to 38% in water. Figure 17 shows the relative difference in soluble copper measured for each copper source alongside sulphur combinations in rumen fluid. In this trial, the soluble sources ($P<0.0001$) still had the highest solubility (42-49±5-16%). Copper acetate and copper carbonate were ($P<0.0001$) less soluble than the soluble sources but were significantly higher than tribasic copper chloride and copper oxide which were least soluble (11±8% and 8±1% respectively).

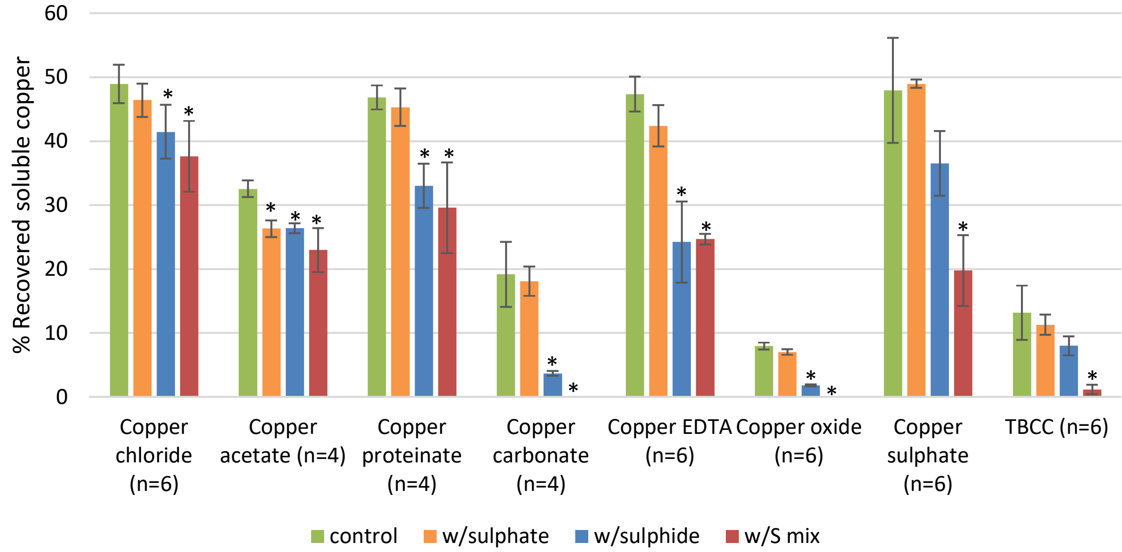

Figure 17: Percentage soluble copper for each source in comparison with baseline solubility in rumen fluid (left bar, control) compared to sulphur sources; w/sulphate, w/sulphide and w/S mix (both sulphate and sulphide). Error bars denote standard error of the mean. Asterisk denotes significant difference for sulphur sources compared to baseline for each copper source.

Sulphide ($P<0.0001$) lowered solubility across all sources in comparison to the baseline, to an average of 22% (±5). This was ($P<0.0001$) higher than the comparable solubility in water (data not shown). The solubility of the individual compounds showed the previously observed pattern. Although, in this test copper EDTA was ($P<0.05$) lower than copper chloride (24±13% and 41±7% respectively). The insoluble sources remained the least soluble ranging from 2-8% (±1-5%) (Figure 17).

The combination of sulphur and sulphide was no different to the effect of sulphide alone. Similar to the effects of sulphide, in this test the rumen fluid had a ($P<0.0001$) higher average solubility (17±6%) in comparison to water (3%) (data not shown). Copper chloride maintained the highest solubility (38±11%) which was ($P<0.05$) higher than all other compounds except; copper proteinate, copper EDTA and copper acetate. All the insoluble sources had 0% solubility, only tribasic copper chloride reported a value which was 1±1% (Figure 17).

Across all the copper sources, the highest proportion (22±15%) of resoluble copper after abomasal-simulation was found when no sulphur source was added. With the addition of sulphate this did not lower significantly (20±13%) (data not shown). Where sulphide was added, and in the sulphur mix treatment, the resoluble copper was ($P<0.0001$) lower; at 13±10% and 9±2% respectively. Resoluble copper was least in sulphur mix with iron (6±5%). This was ($P<0.001$) less than all the other treatments except sulphur mix alone. Copper chloride measured ($P<0.0001$) more copper (27±21%) across the different sulphur treatments than all the other sources. Tribasic copper chloride (TBCC) measured the least resoluble copper (8±4%); ($P<0.05$) less than copper chloride and copper sulphate which measured 18±12% (data not shown).

4.4.3 Effect of iron on copper solubility

When iron was included in the experimental design, the effects found were similar to tests without iron. Where iron was added to copper chloride followed by relevant sulphur source, no additional effect from the iron was observed in any of the tests. The solutions without sulphur had the highest soluble copper (65±6%, 63±3%, without and with iron respectively), followed by sulphate (62±2%, 60±4%), sulphide (49±5%, 51±3%) and sulphur mix (37±0%, 44±9%) which had the least soluble copper. The sulphur mixes with and without iron were ($P<0.05$) lower than the solutions without sulphur and with sulphate. No other effects from the iron were observed (Figure 18).

The average solubility across all compounds did not differ for either sulphide treatment (sulphide and sulphur mix). There was an ($P<0.0001$) increase in solubility in rumen fluid compared to water (19% compared to 5%). In rumen fluid, copper sulphate had the highest solubility (39±6%) where iron was present alongside sulphur mix; which was ($P<0.0001$) higher than all other compounds except copper proteinate and copper chloride (Figure 18). In this trial, copper sulphate had a ($P<0.05$) higher solubility with iron than without it. The least soluble sources were those insoluble in water, with iron and sulphur mix present tribasic copper chloride had the highest solubility at 2±2%. Copper carbonate was completely insoluble in this test.

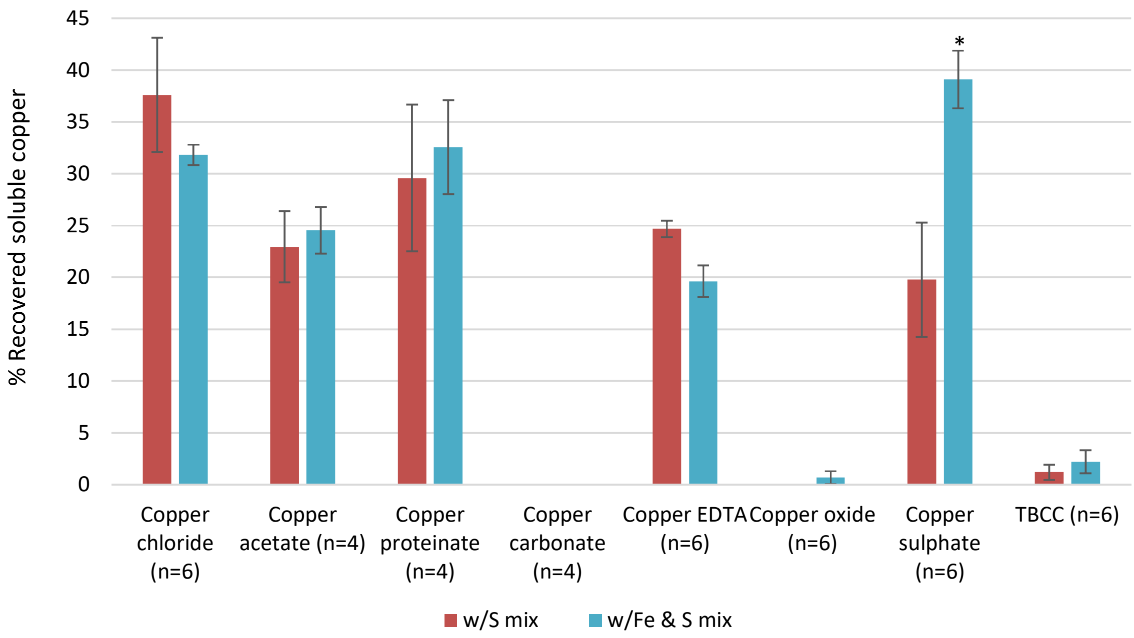

Figure 18: Percentage soluble copper for each source with sulphur mix vs sulphur mix with iron. Error bars denote standard error of the mean. Asterisk denotes significant difference (P<0.05).

4.4.4 Effect of molybdenum on copper solubility

When no sulphur source was added the element combinations which also contained molybdenum had (P<0.01) higher copper solubility (~70%) than those which did not contain molybdenum (~60%). The tests which contained sulphur sources showed no difference in copper solubility between any of the combinations (Figure 19). Across the elemental combinations the previously observed effects of sulphide were evident on copper solubility (P<0.0001).

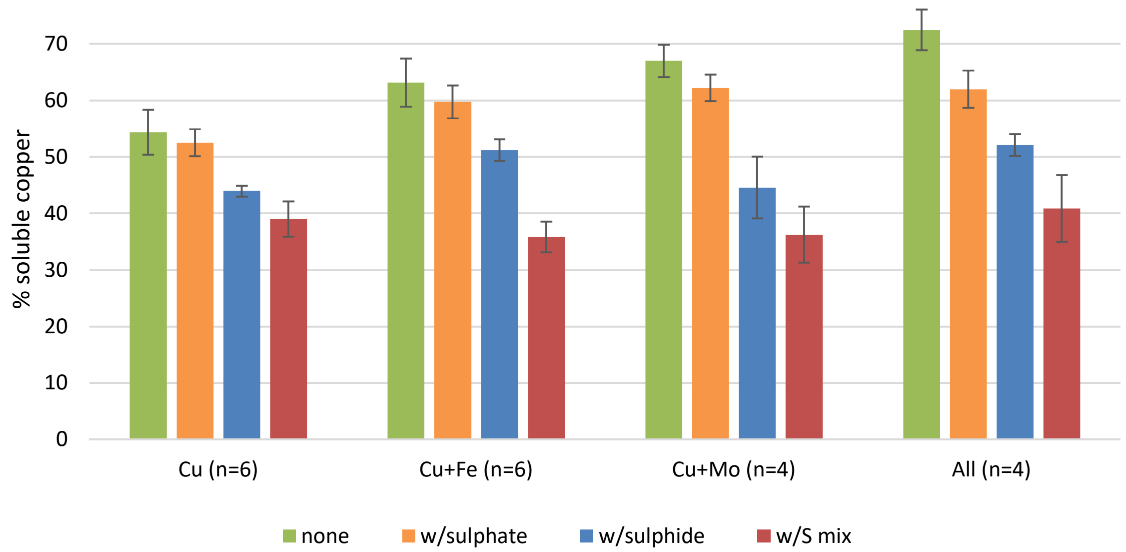

Figure 19: Percentage soluble copper from copper chloride for different element combinations (Cu+Fe, Cu+Mo, Cu+Fe+Mo) in comparison with baseline solubility (Cu) in rumen fluid (left bar) and with different sulphur sources. Error bars denote standard error of the mean.

Copper without iron or molybdenum measured the highest percentage ($P<0.05$) of resoluble copper across all the sulphur treatments (32±21%) after abomasal-simulation. The least resoluble copper was measured from treatments containing the sulphur mix (5±4%) irrespective of the other elements present (data not shown).

4.5 Discussion

The baseline solubility of copper appeared lower in rumen fluid than in water across all the copper sources tested; indicating the strength of competing ligands present in the rumen fluid and the impact of the reducing environment on the simple copper chemistry. Similar to the water trials, copper sulphate, copper chloride, copper acetate, copper EDTA and copper proteinate remained the most soluble in rumen fluid. After abomasal-simulation, an additional proportion of copper became available for each copper source. The insoluble sources in this trial were; copper carbonate, copper oxide and tribasic copper chloride (TBCC) which were consistent with the data using water in Chapter 3. These insoluble sources measured the largest relative proportions of resoluble copper in comparison to their baseline solubility. In the case of TBCC the combined total soluble copper from both soluble and resoluble copper totalled a larger percentage than the soluble percentage in water. However, for each of the sources the solubility only rose up to ~30% which remained lower than the soluble copper sources. These findings support the evidence that copper oxide will remain insoluble after acid digestion in the abomasum, resulting in feed-grade copper oxide remaining insoluble and therefore essentially unavailable under normal digestive processing (Kegley and Spears, 1994; Spears, 2003). Tribasic copper chloride (TBCC) measured twice as much soluble copper in rumen fluid from both the soluble and resoluble fractions combined than the baseline solubility in water, but remained <25% soluble. Unlike previous work in silage-fed steers, where TBCC was measured as equally soluble to copper sulphate under abomasal conditions copper sulphate maintained a higher solubility in the rumen fluid (Genther and Hansen, 2015). The lack of solubility in water and rumen fluid at ruminal pH is in line with the theory that this source is able to by-pass the rumen. However, the theory continues to indicate that solubility will be increased under the acidic conditions of the abomasum (Miles *et al.*, 1998; Spears, 2003, 2013; Arthington, 2015). This latter part of the theory was not sufficiently supported by the current data since the total solubility remained very low in comparison to all the soluble sources.

As anticipated, copper carbonate had low solubility in water and rumen fluid. Although, previous studies have found that its solubility increased up to ~70% after incubation with hydrochloric acid (Ward *et al.*, 1996). The experimental design used in the previous work was comparable to that used in the present study. However, the concentration of hydrochloric acid used was 0.03 M; three times stronger than that used in the present experiment to replicate abomasal conditions (Koritz, 1982; Cao *et al.*, 2000; Moran, 2005). Additionally, the incubation period was either 2 h or 24 h, both contact times exceed the parameters used to replicate abomasal retention time in this work of 1 h. The increased concentration of acid and incubation time could easily account for the increase in solubility established in the previous work. However, the post-ruminal availability of copper chloride and TBCC has not been effectively demonstrated in the present trial and may be lower than previously thought.

In previous animal trials, copper sulphate was found to be more available than copper chloride (Ledoux *et al.*, 1995), although, these were not antagonist trials and no data was given on the sulphur content of the basal diet making comparison more difficult. Interestingly, the present work contrasts this previous work as copper chloride appeared to retain the most consistent and the highest solubility. Copper acetate has been demonstrated to have a similar bioavailability to copper chloride in sheep (Ledoux *et al.*, 1995), but in all the present trials was notably less soluble, and therefore potentially less available.

The nature of ligand bonding in organic copper sources are theorised to be more bioavailable and to protect copper from rumen interactions (Hansen *et al.*, 2008; Spears, 2013). However, in the present work, the organic sources did not remain more soluble than the ionic sources under antagonist challenge from sulphide or iron. Copper proteinate performed similarly to copper chloride and sulphate which is also the case in other feed trials (Wittenberg *et al.*, 1990a). While copper acetate sustained the lowest solubility of the soluble copper sources throughout the work. The present work also found copper EDTA to be highly soluble which is consistent with work suggesting it may be highly bioavailable and similar to copper sulphate (Suttle and Price, 1976; Ammerman *et al.*, 1995).

The relative estimated bioavailabilities for each of the copper sources tested compiled as averages from available literature data are shown in Table 4 alongside estimated solubility after ruminal and abomasal simulated digestion from the current work.

Table 4: Average estimated bioavailability of copper sources from literature relative to copper sulphate and estimated solubility relative to copper sulphate in the current work. Compiled from rumen fluid soluble and resoluble baseline data, ± standard error (Wittenberg *et al.*, 1990a; Ledoux *et al.*, 1995; Jongbloed *et al.*, 2002; Spears, 2003).

Copper source	Average relative bioavailability from literature	Relative solubility from current work
Tribasic copper chloride	115%	44% ±7
Copper proteinate	102%	89% ±3
Copper sulphate	**100%**	**100% ±13**
Copper chloride	100%	111% ±15
Copper acetate	100%	71% ±6
Copper EDTA	95%	94% ±8
Copper carbonate	93%	51% ±8
Copper oxide	42%	37% ±4

It has been suggested that the solubility of copper in water or physiological fluid is a good indicator of bioavailability and digestibility (Krupanidhi *et al.*, 2008). However, the results from the current work show a different pattern of potential availability to that estimated from the literature. Part of this variation may be due to the inherent differences in experimental design. Data compiled from literature has been estimated using largely *in vivo* work and has used different parameters to determine bioavailability such as; plasma and liver copper and caeruloplasmin, discussed in Section 3.1. Several studies have focussed on applying data found *in vitro* models to predict *in vivo* bioavailability, but there is not always a correlation between the two, due to the complex interactions which struggle to be replicated *in vitro*; and in some cases, highly soluble sources *in vitro* appear to have little bioavailability to the animal (Richards, 2010). It is also difficult to replicate and account for absorptive losses between the two contrasting designs. In general, techniques that rely on chemical assays and *in vitro* methodology are of limited utility and can potentially generate misleading results and as such, are not directly comparable (Richards, 2010). Despite this discrepancy, copper sulphate, copper chloride, copper proteinate, and copper EDTA all appear to be readily available sources across both estimations, and although much less available, copper oxide also gave similar averaged values across both estimations. Copper carbonate, copper acetate and TBCC all appeared to be less available in the current work than average estimates *in vivo*.

Molecular characteristics have a potential role to play where different bioavailability values for soluble sources are observed *in vivo* in comparison to solubility work. Differing molecular size limits exist in different regions of the intestine (Kostal, 2016). In general, as molecular size increases, bioavailability decreases (Kostal, 2016). It is considered that molecules with a molecular weight in excess of 1,000 Da have negligible bioavailability (Kostal, 2016). However, other measures exist to determine the absorbability of a molecule such as their molecular volume, often used in medicine to determine intestinal absorption; molecular shape and molecular surface area (Kostal, 2016).

Solubility is paramount to bioavailability but does not equate to the same parameter. Therefore, the results of this work only give a simplistic representation of potential bioavailability rather than a bioavailability measurement and complexities within the rumen and systemic environment may account for an increase in bioavailability in the animal which cannot be seen in the rumen fluid solubility model.

Data from the current work demonstrates sulphur's ability to reduce copper solubility in the rumen. The effect of sulphate was similar in both water and rumen fluid and made no significant decrease on copper solubility; supporting the notion that sulphide is required for interaction (Standish and Ammerman, 1971; Rosa *et al.*, 1986). The most pronounced effects of this were observed in the sulphide treatment and sulphur mix, which comprised biological concentrations of both sulphate and sulphide. With this treatment, copper solubility was reduced by as much as 30% from its baseline. The appearance of this pattern across both substrates also supports the theory that this interaction is purely chemical in nature and as a result is likely to be restricted to the digestive tract. Where the action of the sulphur-reducing bacteria in the rumen create ruminal sulphide which reacts with available copper forming insoluble copper sulphide, which remains insoluble even under acidic conditions (Mills, 1960; Bird, 1970; Bird and Moir, 1971; Suttle, 1974a; Ivan, 1988; Mountain and Seward, 1999). The continued insolubility of copper sulphide is also supported in the current work by the low resoluble copper values where sulphide rather than sulphate or no sulphur source was present. Supporting the notion that insoluble copper complexes are stable and once formed remain intact even at lower abomasal pH (Ivan and Veira, 1981). The current work adds further evidence to support the potential for the effects of sulphur alone to impose a significant reduction on copper solubility in ruminants. Although in a dynamic biological matrix with competing ligands present this may not contribute as strongly to the overall antagonism, but it may participate more greatly than previously anticipated.

Interestingly, the percentage of soluble copper was altered where iron was also added to sulphur mix, although across all the sources this difference was not significant. Copper solubility ($P<0.05$) increased with the presence of iron for copper acetate, copper proteinate, copper sulphate and tribasic copper chloride; increasing by 19% in the case of copper sulphate; and increasing on average by 7% across these sources. This unexpected effect of iron on the effect of sulphide may show some indication of a protective effect.

Soluble iron was ($P<0.001$) higher in sulphur mix without copper (8%) than in the sulphur mix with copper sources (7%). This could potentially be an indication of copper-iron-sulphur complex formation. Although, this cannot be considered evidence and effects here are weakly indicated and inconsistent requiring an *in vivo* model to be further studied.

The present results did not show a significant decrease in soluble copper where molybdenum and sulphur were added. Copper solubility remained between 30-40% in the sulphur mix treatments irrespective of the presence or absence of molybdenum. However, soluble molybdenum was ($P<0.0001$) reduced where copper was also present when compared to molybdenum without copper. Molybdenum solubility was reduced from 74% across all the sulphur treatments to 63% in the presence of copper. The presence of sulphur also had a marked effect on molybdenum solubility ($P<0.0001$) reducing solubility from ~74% with sulphate and without a sulphur source, down to ~60% with sulphide and with the sulphur mix. The reduction in soluble molybdenum present can potentially be attributed to the formation of thiomolybdate. Suggesting that a percentage of copper-thiomolybdate may have formed *in vitro,* although, this could not be differentiated from copper sulphide formation in this trial. An *in vitro* study of similar design by Goselink (2014) found that where molybdenum and sulphur were present none of their samples had a copper concentration above the ICP-AES detection limit of 500 µg/l. The authors attributed this to an almost complete binding of copper through a high concentration of thiomolybdate formation. Previous work has established that thiomolybdates are able to form in rumen fluid under replicated rumen conditions similar to those in the present work (Mills *et al.*, 1977; Clarke and Laurie, 1980; Bray *et al.*, 1982a). When molybdate and sulphide were mixed in non-fermentive rumen conditions tetra-thiomolybdate was formed at S^-:Mo ratios >10:1 (Bray *et al.*, 1982a). In the present work a ratio of S^-:Mo of 30:1 was used which should be sufficient to elicit formation. However, only around 10% of the added molybdenum appeared to become insoluble. The work conducted by Goselink (2014) only introduced a sulphate source making it difficult to determine the concentration of sulphide provided in their trial. However, a maximum conversion of sulphate into sulphide of 97 mg/l (Anderson, 1956; Bailey and Balch, 1961), would provide a ratio >2000:1 therefore any conversion of sulphate into sulphide over 2% would create a ratio sufficient to elicit thiomolybdate formation in their work, potentially explaining the difference in copper binding found between the two trials.

The effect of sulphide was the most potent effect on copper solubility observed on an *in vitro* basis and without a further decline in copper solubility where molybdenum was also present it is not conclusive in the current data whether copper-thiomolybdate formation took place.

4.6 Conclusion

The rumen simulation of solubility gave similar results to those in de-ionised water (Chapter 3). Although, the solubility of each copper compound in rumen fluid was generally lower the same pattern of solubility across the sources was still present. The abomasal-digestion simulation had less soluble copper than expected; especially for copper carbonate and TBCC. The hierarchy of bioavailability appears similarly variable in laboratory trials as *in vivo* work. There are commonalities between the two modes of study which may make them useful predictors, but only in combination with *in vivo* testing.

The ability of sulphur, as sulphide to reduce copper solubility observed in de-ionised water (Chapter 3) was also evident in rumen fluid. The interaction which takes place between iron, copper and sulphur is not purely chemical in nature and it cannot be effectively replicated using *in vitro* conditions. This model was also inconclusive in detecting the effects of thiomolybdate formation from the effects of copper sulphide formation. The apparent starting point for the study to look at the effects of the antagonists at a chemical level has determined that there is a factor intrinsic to the rumen environment which participates or catalyses these reactions. The nature of the iron interaction *in vivo* is not obvious from this current work, and cannot be easily replicated under laboratory conditions. Further elucidation of the nature of this antagonist pathway must continue *in vivo.*

5.0 Copper binding and movement through digestion

5.1 Introduction

Work modelling the antagonist pathways *in vitro* has shown that the complexity of the rumen cannot be easily replicated. To further understand the antagonist modes of action, mapping the movement of copper and its antagonists through digestion will be useful to relate the potential modes of action to the observed clinical effects.

5.1.1 Thiomolybdates in digestion

Within the rumen environment thiomolybdates are known to associate with protozoa, bacteria and un-digested feed particles within the solid phase of the rumen fluid (Price and Chesters, 1985). Here, they form a more stable and more potent ligand when reacted with copper (Allen and Gawthorne, 1987; Price *et al.*, 1987; Gooneratne *et al.*, 1989a; Suttle, 1991; Gould and Kendall, 2011). The formation of thiomolybdates associated with the solid rumen digesta act as temporary intermediates, which then interact with the solid phase constituents and form powerful chelators with a strong affinity for copper (Allen and Gawthorne, 1987). The copper-thiomolybdates bound to solid phase particulate are able to pass through the rumen into the abomasum without being degraded and do not release copper even under acidic conditions (Campbell *et al.*, 1974; Allen and Gawthorne, 1987; Price *et al.*, 1987; Suttle, 1991). Thus, making it unlikely that these complexes would allow absorption of copper in the intestine; reducing the amount of bioavailable copper (Gooneratne *et al.*, 1989a). This lack of absorption is supported through the finding of copper-tetra-thiomolybdate products in the faecal material of animals fed molybdate-rich diets (Suttle, 1991). Through the use of radio-labelled molybdenum, it has been demonstrated that thiomolybdates associate with the solid phase and potentially only appear in the liquid phase of the rumen fluid once the solid phase has reached saturation (Price *et al.*, 1987). Differences in copper metabolism due to forage type may be linked to the solid phase association of thiomolybdate. Where corn-silage diets, expected to produce little solid phase, showed no effect on DMI and milk yield in cattle fed a high sulphur and molybdenum diet, their grass-silage fed counterparts did; although the process by which this takes place is not well understood (Suttle, 2010; Sinclair *et al.*, 2017).

5.1.2 Systemic thiomolybdate effects

When supplied in the diet, thiomolybdate inhibits copper absorption into the systemic circulation, but when provided in the absence of dietary copper, or through a parenteral route, thiomolybdate in the bloodstream forms an albumin complex rendering serum copper unavailable for cellular uptake (Crisponi *et al.*, 2010). The main carrier of thiomolybdate in the blood stream is albumin (Wang *et al.*, 1992). Once in circulation, thiomolybdate can bind reversibly to both bovine and ovine serum albumin (Mason *et al.*, 1982; Hynes *et al.*, 1984). The binding of thiomolybdate takes place in bovine serum albumin at a single site, separate from the copper binding site (Woods and Mason, 1987). When copper is also bound to the albumin-thiomolybdate complex, the resulting complex seems to be relatively stable and not subject to hydrolysis or excretion (Hynes *et al.*, 1984; Wang *et al.*, 1992). In addition to complexation with albumin, absorbed thiomolybdate can associate with other proteins, such as metallothionein in the liver (Wang and Mason, 1988). These protein-bound thiomolybdates are able to act as a powerful copper ligands, which alter the distribution of copper in the tissues and interfere with copper regulatory mechanisms (Wang *et al.*, 1992; Laurie, 2000).

The cuproenzymes and copper proteins; caeruloplasmin, metallothionein, CCO, SOD, tyrosinase, anti-oxidant 1 (Atx1) and ascorbate-oxidase, are all known to be inhibited through binding with thiomolybdate (Chidambaram *et al.*, 1984; Suzuki *et al.*, 1995). The mechanism by which thiomolybdate crosses the cell membrane and binds to the biological copper components remains unknown (Clarkson *et al.*, 2019), but it disrupts copper transport through the formation of stable thiomolybdate-copper clusters; binding to the copper from chaperones and copper-enzymes without removing the copper component (Suttle, 2012). Some studies suggest that the effects on SOD may differ, and the copper may be partially stripped from the enzyme in this case (Juarez *et al.*, 2006, 2008). X-ray absorption has been used to establish the mode of thiomolybdate bonding to biological copper compounds. This method has been used to determine the oxidation state of copper and molybdenum within the Atx1-thiomolybdate complex as Cu^{+} and Mo^{6+} (Alvarez *et al.*, 2010). The Atx1-thiomolybdate molecule has few hydrogen bonds between the monomers and is held primarily by co-ordinate bonds between sulphur-cysteine (CysS) complexes. At the centre, lies a copper-molybdenum cluster containing four Cu^{+} atoms, a single MoS_4^{2-} cluster and three pairs of Atx1-CysS complexes creating a $[S_6Cu_4MoS_4]$ cluster (Alvarez *et al.*, 2010). This structure demonstrates that the intracellular sequestering of copper from its chaperones involves thiomolybdate supressing the incorporation of copper into the enzyme and disrupting its activity through appropriating the copper without removing it (Alvarez *et al.*, 2010). Thus, revealing the potential for even a small amount of thiomolybdate, if not complexed with copper in the rumen and eliminated, to disrupt the transport and dissemination of copper through the suppression of copper trafficking on an intracellular level (Clarkson *et al.*, 2019).

5.1.3 Iron complexing in digestion

The iron antagonism and its resulting compound is much less understood. The evidence for this antagonism is predominantly from animal trials; discussed in Section 1.8 (Humphries *et al.*, 1983; Bremner *et al.*, 1987; Phillippo *et al.*, 1987b; Prabowo *et al.*, 1988; Mullis *et al.*, 2003; De Sousa *et al.*, 2012). These trials focussed on measurement of liver and blood parameters. While this effectively demonstrates the existence and components involved in this pathway it can only pose theoretical hypotheses for the mode of action and compound formed.

The only trial which has investigated internal flow and solubility of both copper and iron was Ivan, Ihnat and Veira, (1983). This novel experimental design used re-entrant cannulae in the small intestine to determine concentration and apparent absorption from different silage based diets. While the results of this study indicated the main site of absorption for both of these elements is the ileum and that there are effects of diet on apparent absorption, such as increased iron absorption from cereal based diets and increased copper absorption with the inclusion of urea at ensiling. The differing diets were not designed to investigate the effects of antagonists on the movement of copper or to investigate the changes in solubility which occur earlier in digestion.

There is still much to be learned about the way in which copper moves through ruminant digestion and how this movement is affected by the presence of antagonists.

5.1.4 Lixiviation of minerals using water

Lixiviation is a process where soluble and insoluble substances are separated by dissolving the soluble component in water, or other solvent. This technique has been applied in geology and soil chemistry since the 1970s to determine the potential availability of trace minerals in soil (Cronan *et al.*, 1978; Miller, 1979). More recently, it has been used to determine the concentration of soluble salts in pigeon droppings (Gómez-Heras *et al.*, 2004), but has not yet been used on ruminant faecal material. Using this technique on ruminant faeces has the potential to discover the percentage of copper, iron and molybdenum which is able to be solubilised through surface contact and dissolution into a non-mineral containing water column; which can then be compared to the full elemental composition. Use of this technique could help to determine the percentage of minerals which have been egested that still remain water soluble and therefore potentially bioavailable.

5.2 Aims & Objectives

The aim of this work was to trace the movement and solubility of copper through digestion with and without antagonist challenges to observe any differences and to determine the effects on short-term copper status through monitoring blood parameters. A secondary aim was to determine the percentage of copper which remains water soluble; as a measure of potential bioavailability in the faeces of animals fed varying antagonist diets.

5.3 Materials & Methods

5.3.1 Animal experimental design

Four 5 year old, non-lactating, adult female Holstein-Friesian cows (BW $\bar{x}$ 732±20 kg) at maintenance with rumen fistulae received the experimental basal diet comprising 2 kg/day formulated compound feed (Table 5) and 10 kg/day (±2 kg/day) short-chopped wheat straw for a period of one week prior to the trial start date (Table 6).

Table 5: Compound feed ingredients and percentage inclusion

Ingredient	Maize	Soyabean meal	Cane molasses	Salt (NaCl)	Salt (CaP)	Salt (MgO_2)
Percentage	39.75	50	5	2.5	2	0.75

Straw ration was calculated and provided at 100% of previous intake averaged over a fortnight. Basal diet was measured for mineral content of copper, iron, molybdenum and sulphur (Table 5). During the trial, animals were housed indoors on tie-stall cubicles for a total of 4 weeks. Cubicles were furnished with rubber mattresses and cleaned at least twice daily. Ventilation, daylight and temperature were all controlled to ambient conditions. Experimental work was conducted in the Netherlands under DEC (Dutch Ethics Committee) approval number 20400 and UK ASPA (Animals (Scientific Procedures) Act 1986) non-UK 00010 approval.

Table 6: Mineral content of the basal diet for controlled elements as analysed by ICP-MS ± standard deviation.

	Iron mg/kg DM	Molybdenum mg/kg DM	Copper mg/kg DM	Sulphur g/kg DM	% of total diet
Wheat straw	21±4.1	0.4±0.2	2.4±0.8	0.7±0.05	83.3
Compound feed	352±4.31	2±0.07	22.9±0.7	3±0.2	16.7
Concentration in complete basal diet	**76.1**	**0.7**	**5.8**	**1.1**	**100**

The experimental basal diet was fed throughout the trial. Compound feed was first provided each morning and once eaten, straw provided as forage in two 5 kg meals. Leftover straw was weighed and recorded. Waste straw from the floor was also collected and weighed to account for loss in the ration.

Animals were randomly allocated to a treatment group using MS Excel (2013, Microsoft, Washington, USA). Treatments were allocated to random blocking and colour coded for blinding purposes. The treatment groups conformed to a 4x4 Latin square design. Treatment groups consisted of; $Mo^{++}Fe^{+}$, Mo^{+}, Fe^{+} and 'none' (Table 7). Treatments comprised reagent grade sources; copper chloride (VWR International Ltd, Lutterworth, UK), sodium sulphate (VWR International Ltd, Lutterworth, UK), sodium molybdate (Fisher Scientific, Loughborough, UK), and iron chloride (Fisher Scientific, Loughborough, UK) mixed into solid and liquid phase markers titanium dioxide (50 g/day, VWR International Ltd, Lutterworth, UK) and polyethylene glycol (PEG mw 6,000, 20 g/day, Sigma-Aldrich, Dorset, UK) as binders which were all weighed into 250 g polypropylene bottles. During production, a weighing error resulted in a 10-fold increase in molybdenum concentration creating the $Mo^{++}Fe^{+}$ treatment.

Table 7: Total concentration of controlled minerals, treatment dose and markers added through fistula. Asterisk denotes production error resulting in 10-fold dose increase for this treatment from original design.

		$Mo^{++}Fe^{+}$	Mo^{+}	Fe^{+}	None
Iron mg/kg DM	Treatment dose	371	0	371	0
	Total	**450**	**79**	**450**	**79**
Molybdenum mg/kg DM	Treatment dose	11.3*	1.3	0	0
	Total	**12***	**2**	**0.7**	**0.7**
Copper mg/kg DM	Treatment dose	4			
	Total	**10**			
Sulphur g/kg DM	Treatment dose	2			
	Total	**3**			
Titanium dioxide g/day	**Total**	**50**			
PEG 6,000 g/day	**Total**	**20**			

Animals were fed from 6:00 am each morning. Fifteen minute intervals were left between animals to allow time for sampling to take place (Figure 20). Once each animal had received their compound feed their allocated treatment in the form of a mixed powder was placed directly into the rumen fistula every morning for 5 days. On day 4 and 5, samples were collected pre-treatment (0 h) and at 3, 7 and 11 h post-treatment. Animals had 2 rest days where basal diet was provided without treatment prior to the start of the next trial period (Figure 20).

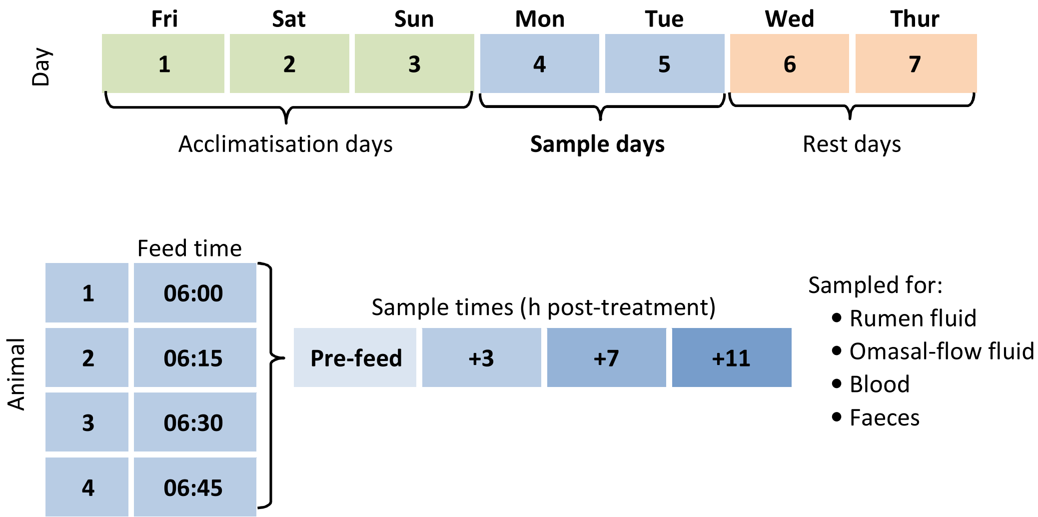

Figure 20: Outline of trial period acclimatisation, sample and rest days and time points used for feeding and sampling.

Unfortunately, the final data set was incomplete as one of the animals was withdrawn due to a reduced DMI during the experimental period (discussed in Section 5.5.2).

5.3.2 Sample collection

5.3.2.1 Faeces

Faecal samples were taken from the rectum using arm length gloves with mineral-free lubricant (Agri-gel, Net-Tex, Staffordshire, UK) to minimise discomfort. Animals were stimulated to defecate using rectal manipulation and faecal grab method where required. Faeces which had contacted the lubricant was discarded and a ‘clean’ sample (~300 g) was collected into a foil tray and frozen at -20°C for analysis.

5.3.2.2 Rumen and omasal-flow fluid

Samples of rumen and omasal-flow fluid were collected via the rumen fistula, using bespoke sampling lines which were designed for the purposes of this trial. Preliminary tests to optimise the design of sampling devices for rumen and omasal fluid collection and to refine the standard operating procedures for their use were conducted from 6 weeks prior to the trial start date. This process confirmed the devices were fit for purpose and did not cause discomfort to the animals, and also provided an estimation of the time required and ease of sample collection, giving a more accurate reflection of sample point duration for each animal. A schematic of the sampling devices is shown in Figure 21.

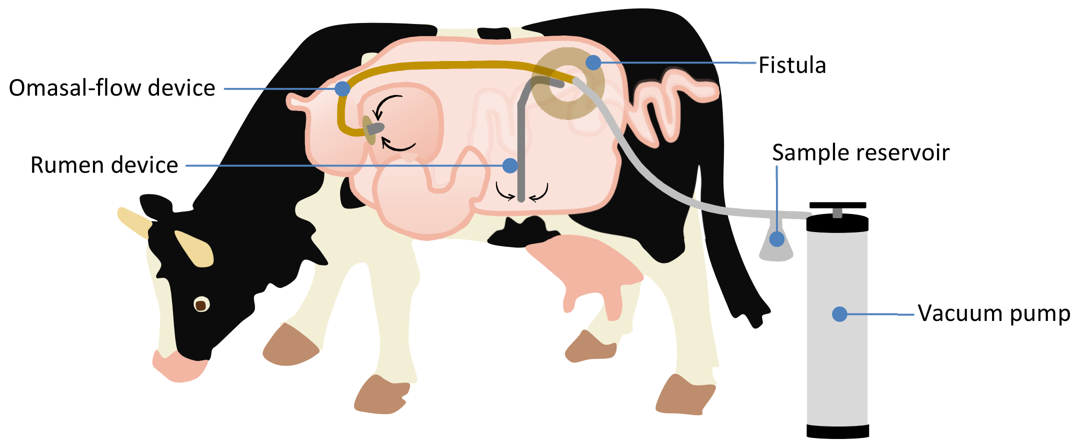

Figure 21: Schematic of the sampling lines and devices for rumen fluid and omasal-flow fluid *in situ*.

Samples were collected by attaching a vacuum pump (RS Pro, Northants, UK) to a customised polypropylene tube placed into the rumen or omasum, without requiring the fistula to be opened for sampling. Rumen samples were obtained through an ⅛" inner diameter (id) open-ended flexible polypropylene tube which was introduced into the rumen at each sample point through a larger (½" id) fixed tube with a closed end and pores to allow fluid to pass through but prevent blockage from larger particulate. The outer fixed tube was adapted to the rumen fistula and measured for each animal with the aim of ensuring a consistent sample point within the rumen. The fixed tube was positioned *in situ* for each sample and removed and cleaned after sampling. To collect samples, one end of the flexible inner tube was threaded into the positioned fixed tube and the free end attached to the vacuum pump. Upon application of the vacuum, rumen fluid flowed through the inner tube. Once fluid was flowing, the vacuum was removed and the collected reservoir of fluid used to fill (~10 ml) 2 x 14 ml polypropylene collection tubes (Sarstedt Ltd., Leicester, UK). Inner tube lines were inserted for sampling and removed and washed after each sample point using clean, potable water and air dried.

Duplicate samples (2 x ~10 ml) were also taken from the omasal junction using a similar tubing technique. For the omasal-flow samples, the outer tube consisted of a more flexible length of ½" id polypropylene tubing with a flexible silicone phalange and caged end placed in through the fistula so that the tube was held in place by the phalange at the reticulo-omasal orifice. This tube was placed *in situ* before the start of the trial. Each day, the sample line was checked for blockages by drawing fluid into a clean inner tube and returning it. The outer tube was only removed or adjusted if blockages occurred to minimise discomfort to the animal. The inner tube conformed to the same procedure as the rumen tube and was introduced and removed for cleaning with water at each sample point.

Both rumen and omasal-flow samples were centrifuged at 2,000 *g* for 15 minutes (Rotina 380, Hettich instruments, Beverly, USA) to allow some of the larger biota and colloidal particles to sink to the bottom of the tube prior to freezing and storage at -20 ˚C. No separation or fractionation was made to the fluids at this point.

5.3.2.3 Blood

Blood samples were collected from the coccygeal vessels of the tail vein using an 18 gauge needle (Becton-Dickinson, Winnersh, UK) and needle holder into 2 x 10 ml lithium-heparin (LH) and 2 x 10 ml no additive (EST™) tubes (Vacutainer®, Becton-Dickinson, Winnersh, UK). Blood tubes were inverted ~10 times to ensure thorough mixing. LH tubes provided two replicates of 1.8 ml samples of whole blood pipetted into cryotubes (VWR International Ltd, Lutterworth, UK) which were frozen (-20 ^{o}C) prior to subsequent analysis. EST™ and LH tubes were then centrifuged (1,500 *g* for 15 mins, Rotina 380, Hettich instruments, Beverly, USA) and duplicate samples of 1.8 ml of both serum and plasma pipetted (Single channel pipette, Adjustable 0.5-5 ml, Eppendorf Research®, Stevenage, UK) into cryotubes (VWR International Ltd, Lutterworth, UK) from LH and EST™ tubes respectively prior to freezing at -20°C.

5.3.3 Sample analysis

5.3.3.1 Digestive fluid separation

Fully thawed rumen and omasal-flow samples were centrifuged for a second time at 2,300 *g* for 15 mins (Allegra X-22, Beckman Coulter, High Wycombe, UK). The supernatant was pipetted (Pasteur 3.3 ml Micro Bulk Wrap Pipette, Scientific Laboratory Supplies Ltd, Nottingham, UK) into a 14 ml polypropylene tube (Sarstedt Ltd., Leicester, UK) and a 500 µl sample taken to analyse through ICP-MS. The remaining colloidal fraction was subject to freeze-drying and wet-acid digestion using the technique described in Section 5.3.3.2. Soluble fractions as a percentage of total elemental concentration from digestive fluid were calculated using the method described by Caldera *et al.*, (2019).

5.3.3.2 Wet-acid digestion

Total elemental composition of solid samples (faeces, rumen colloid and omasal colloid) were determined using ICP-MS following wet-acid digestion. All solid samples were freeze-dried to allow for digestion on a dry matter basis. Weighed and recorded samples of ~1-2 g were placed in a -20°C freezer until fully frozen. Samples were then freeze-dried (Modulyo® M143, Edwards, Leicestershire, UK) for a minimum of 48 h until there was no further weight loss. Approximately 0.1-0.2 g of freeze-dried sample were weighed directly into Teflon microwave digestion tubes (HVT50, Anton Paar, St Albans, UK) and incubated for 1 h with 3 ml 68% HNO_3 (Fisher Scientific, Loughborough, UK), 3 ml deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm), and 2 ml 30% H_2O_2 (Fisher Scientific, Loughborough, UK >99%), then digested for 45 minutes (10 min ramp to 140 ˚C, 20 min hold, then 15 min cooling at 55 ˚C) in a Multi-wave 3,000 microwave (Anton Paar, St Albans, UK), alongside blanks and reference material (certified 1577c, Bovine Liver, National Institute of Standards and Technology, USA and/or an internally cross calibrated standard liver material. intra-assay CV 3.5% and 6.57% for the work in Chapters 5 and 8 respectively). Digested samples were transferred and then washed into universal tubes (Sarstedt Ltd., Leicester, UK) with 7 ml of deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm) and inverted to mix, prior to a 500 µl sample taken for ICP-MS using the procedure outlined in Section 3.3.2.1.

5.3.3.3 Haematocrit

Whole blood was analysed at the time of collection for haemoglobin and haematocrit using electrical conductance of the sample, calibrated, corrected and converted into a haematocrit value using an iStat CG8+ cartridge (Abbott, Princeton, USA).

5.3.3.4 Blood enzyme activity and haemoglobin

Two 25 µl aliquots of mixed whole blood were pipetted from fully thawed cryotubes for analysis of erythrocyte SOD and haemoglobin (Hb) concentrations. Samples were diluted with 1 ml of diluent (RANSEL™ R3, Randox™ Laboratories, Crumlin, UK) and analysed on an RX IMOLA clinical chemistry auto-analyser (Randox™ Laboratories, Crumlin, UK) using RANSOD™ and haemoglobin kits respectively (Randox™ Laboratories, Crumlin, UK), according to manufacturer's instructions (SOD intra-assay CV 6.53%, 4.43% and Hb intra-assay CV 3.13%, 4.67% for the work in Chapters 5 and 8 respectively). The dilutions were modified to allow Hb and SOD to be run from a single common dilution, altered to reflect the common dilution of the reagents. Calibrated results are expressed in relation to haemoglobin.

5.3.3.5 Plasma copper concentration

Thawed plasma was pipetted (500 µl) into a 14 ml polypropylene tube (Sarstedt Ltd., Leicester, UK) for total elemental analysis by ICP-MS using the method described in Section 3.3.2.1.

5.3.3.6 Caeruloplasmin activity

Analysis of serum caeruloplasmin activity was carried out on (~1 ml) thawed serum according to the method of Henry *et al*., (1974), adapted for the RX IMOLA auto-analyser (Randox™ Laboratories, Crumlin, UK). Caeruloplasmin activity was also calculated as a ratio with plasma copper concentration (intra-assay CV 5.69% and 3.41% for the work in Chapters 5 and 8 respectively).

5.3.3.7 Trichloroacetic acid (TCA) precipitation

Thawed plasma (750 µl) was pipetted into a 14 ml polypropylene tube (Sarstedt Ltd., Leicester, UK). An equal volume of 10% TCA solution was added; created from reagent grade TCA (Alfa Aesar, Massachusetts, USA) and deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm). Tubes were capped and bench vortexed until completely opaque. Tubes were left to stand at room temperature for 2 h prior to centrifugation at 1,500 *g* for 15 min (Allegra X-22, Beckman Coulter, High Wycombe, UK) and immediate pipetting of 500 µl of supernatant for ICP-MS analysis (described in Section 3.3.2.1). TCA insoluble copper concentration was calculated by subtracting the plasma copper value from the concentration adjusted value of copper from TCA treated plasma.

5.3.3.8 Water lixiviated copper

Faeces was freeze-dried using the method described in Section 5.3.3.2 and ground using a ceramic pestle and mortar. Between each sample the pestle and mortar were washed with a sequence of; de-ionised water followed by 0.1 M HCl wash, de-ionised water wash, sugar scrub, and a final de-ionised water wash before drying. Each sample (~0.1 g) was weighed out into 14 ml polypropylene tubes (Sarstedt Ltd., Leicester, UK). Using the methods described and evaluated by Gómez-Heras *et al.*, (2004). Deionised water (5 ml) (Purite hp 160, Suez, Thame, UK. 17 MΩ cm) was added to each tube and the tubes capped and inverted to mix prior to placing on a tube roller for 24 h. Tubes were subsequently centrifuged at 1,500 *g* for 15 min (Allegra X-22, Beckman Coulter, High Wycombe, UK) and 500 µl of supernatant pipetted for ICP-MS analysis (described in Section 3.3.2.1).

5.3.4 Digestive marker analysis

5.3.4.1 Turbidimetric analysis for Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) was analysed against a standard curve using an optimised version of the method described by Hyden (1955). Digestive fluid (2 ml) was pipetted into 14 ml polypropylene tubes (Sarstedt Ltd., Leicester, UK) followed by 5 ml of deionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm), 1 ml 0.45 M $BaCl_2 \cdot 2H_2O$ (Alfa aesar, Lancashire, UK), 2 ml 0.15 M (saturated solution) $Ba(OH)_2 \cdot 8H_2O$ (Alfa aesar, Lancashire, UK) and 2 ml $ZnSO_4 \cdot 7H_2O$ 0.175 M (Alfa aesar, Lancashire, UK). Tubes were capped and gently inverted to mix and allowed to stand for 30 min. Tubes were then centrifuged at 1,500 *g* for 8 min and supernatant filtered through a 2.5 µm pore size filter paper (№42, Whatman, Maidstone, UK). Filtered solution (2 ml) was pipetted into a clean 14 ml polypropylene tube (Sarstedt Ltd., Leicester, UK) with 2 ml of freshly prepared precipitate solution comprising; filtered (11 µm pore size) 30% TCA (Alfa Aesar, Massachusetts, USA) in a 4:1 ratio with 0.224 M $BaCl_2 \cdot 2H_2O$ solution and 36 mg/ml gum Arabic (Acros organics, New Jersey, USA). Tubes were left undisturbed for 10 mins and 1 ml of each sample pipetted into a 1.6 ml semi-micro cuvette (Fisher Scientific, Loughborough, UK) and read in batches of 8 in a spectrophotometer (Libra S22 UV/Vis, Biochrom, Cambridge, UK) against a water blank, for absorption using a wavelength of 420 mμ. Sample PEG concentration was calculated using the formula generated from repeated standard curves constructed using rumen and omasal-flow fluid at serial dilutions from 0.25 mg/ml to 0.016 mg/ml.

5.3.4.2Titanium dioxide analysis

Freeze-dried samples (~2 g) of faeces were sent to Masterlab (Trouw, Boxmeer, NL) for titanium analysis using their commercially sensitive protocol.

5.3.5 Data analysis

Data was analysed using a mixed effects model, repeated measures analysis (PROC MIXED, SAS Inst. Inc., Cary, NC). Variables used to group the data into classification levels were; treatment, time, period and animal. Animal was used to comprise the experimental unit. The specified term used in the repeated statements was; time (period), and the subject was the animal. Multiple covariance structures were compared. The AR(1) covariance structure was the most appropriate for this analysis. In the event of a significant treatment and treatment × time interaction, means were separated using the PDIFF option of the LSMEANS statement. Tukey comparisons and paired t-tests were also conducted using Minitab v.18 (Minitab, Pennsylvania, USA) where appropriate. For all response variables, an α value of $P<0.05$ was used as the cut-off for significance. Subsequent increased levels of significance were determined at $P<0.01$, $P<0.001$ and $P<0.0001$. Results are expressed as mean values ± standard deviation. Where no significant difference was found between factors and where mutliple factors have been combined for comparison purposes $\bar{x}$ has been used to denote a summative mean.

5.4 Results

5.4.1 The path of antagonists through digestion

Iron in the rumen fluid supernatant was ($P<0.0001$) affected by time point. The iron in the rumen supernatant showed the pulse-dose appearing at 3 h post-feeding and declining throughout the day (Figure 22a). By design, ($P<0.0001$) more iron was found in the rumen fluid supernatant and colloidal fractions where iron had been added through the treatment (Fe^{+} & $Mo^{++}Fe^{+}$) compared to the non-iron treatments (Mo^{+} & none). Samples contained x̄ 367±243 µg/l and x̄ 265±32 mg/kg DM in the iron-containing treatments combined vs x̄ 177±121 µg/l and x̄ 224±103 mg/kg DM in the non-iron treatments in the supernatant and colloid respectively (Figure 22b). Using the method of Caldera *et al.*, (2019) to establish the soluble percentage, overall iron remained soluble in the rumen fluid with ~90% (x̄ 88±11%) associated with the supernatant (Figure 22c). A ($P<0.001$) higher percentage of the total iron (x̄ 92±4%) was associated with the supernatant in the treatments without added iron than the treatments with added iron (x̄ 84±12%). No significant changes to the percentage distribution of iron occurred over time and no treatment x time interaction was reported for any of the iron parameters.

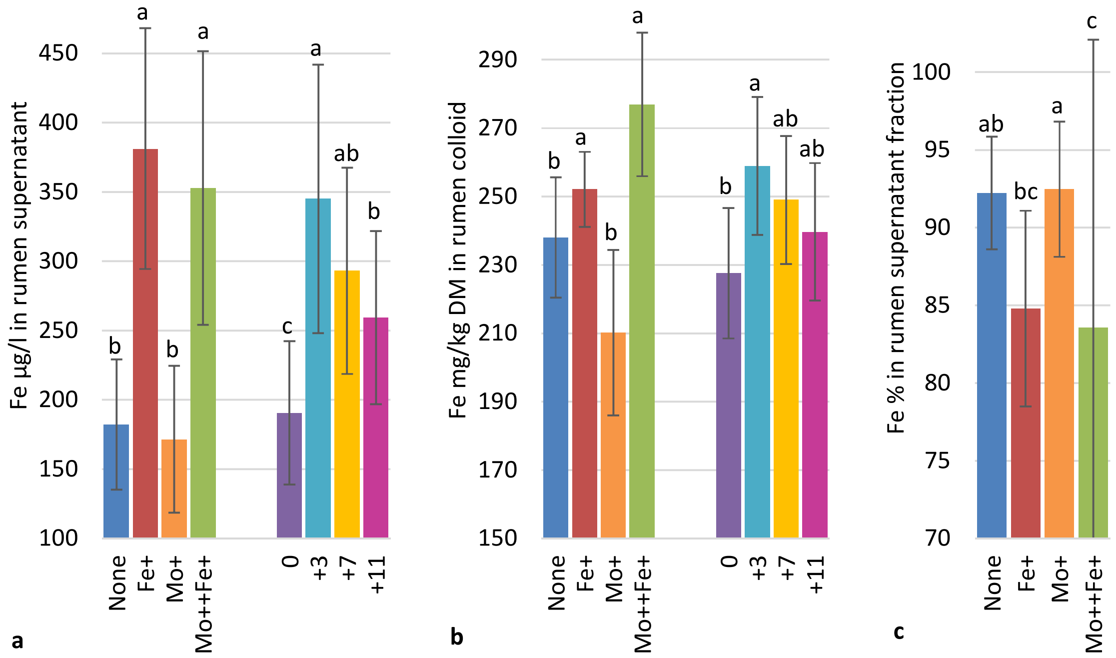

Figure 22: Iron concentration in rumen fluid supernatant (a), colloid (b) by treatment and by time. Percentage of iron in the supernatant fraction (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant (P<0.05) difference by treatment or time.

Iron in the omasal-flow was not affected by time. Iron appeared to be higher in the omasal-flow in comparison to the rumen fluid (P<0.0001). Around 1.5 times more for the colloidal fraction and around 3 times more for the supernatant. More (P<0.0001) iron was found in the omasal-flow supernatant for the $Mo^{++}Fe^{+}$ treatment (1,418±598 µg/l), than the other treatments. Interestingly, in the omasal-flow supernatant there was no difference between iron concentration in Fe^{+} and non-antagonist treatments (833±491 & 736±355 µg/l respectively) while Mo^{+} measured the least (P<0.0001, 493±306 µg/l). In the omasal-flow colloid, there was no significant difference between treatments; although where iron had been added to the treatment measurements were numerically higher ($\bar{x}$ 407±197 vs $\bar{x}$ 353±191 mg/kg DM with iron and without iron respectively). A high percentage of iron remained in the supernatant of the omasal-flow fluid (~80%), which was (P<0.0001) lower than the rumen fluid. A (P<0.0001) higher percentage of iron was found in the supernatant in the Mo^{+} treatment (84±6%) in comparison to the non-antagonist treatment (76±14%) and both had significantly more than the $Mo^{++}Fe^{+}$ treatment (62±18%).

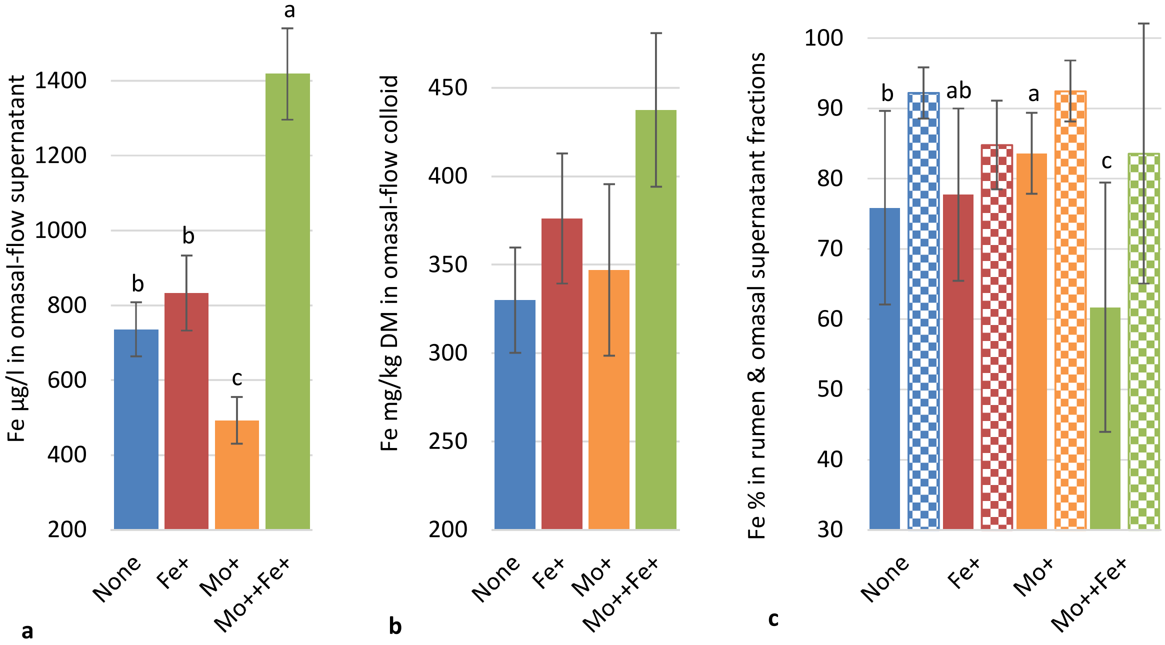

Figure 23: Iron concentration in omasal-flow supernatant (a) and colloid (b) by treatment. Percentage of iron in the supernatant fraction of omasal-flow (solid colour) and rumen fluid (checked colour) (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment. Significant differences for % Fe in rumen fractions previously reported in Figure 22c.

Concentration of iron in the faeces was also as expected from treatment concentrations; where iron was added more iron was reported (Figure 24a). However, this was not significantly different to the non-antagonist treatment. The lowest concentration of iron was in the Mo^{+} treatment ($P<0.01$). After lixiviation, the most iron was found in the Fe^{+} treatment; which was greater than all the other treatments ($P<0.0001$). Both $Mo^{++}Fe^{+}$ and non-antagonist treatments had statistically similar percentages, which were greater than the Mo^{+} treatment (Figure 24b). No time effects were reported for faecal measurements.

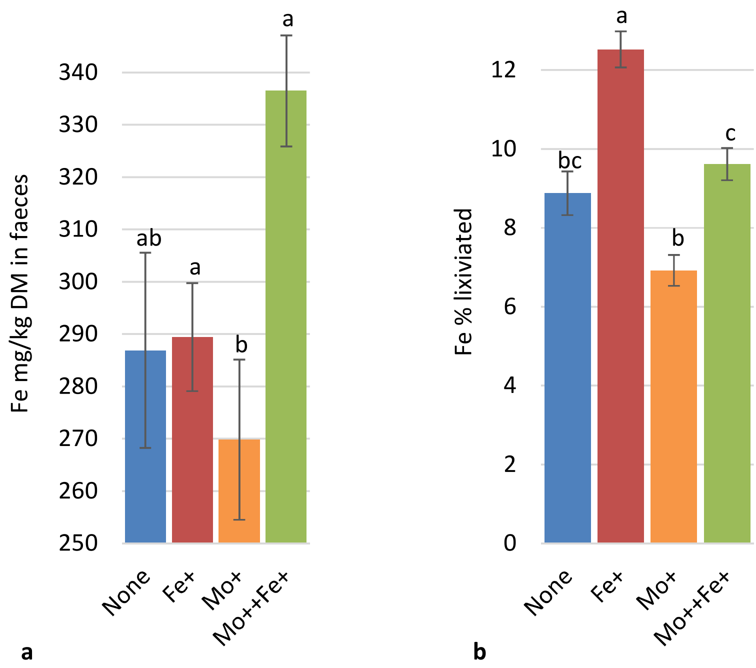

Figure 24: Iron concentration in faeces (a) by treatment. Percentage of iron lixiviated (b) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment.

Molybdenum, like iron, was also ($P<0.05$) affected by time in the rumen fluid; showing the appearance of the pulse-dose and return to basal over time (Figure 25a) and a ($P<0.05$) treatment x time interaction was observed for molybdenum in the rumen supernatant and colloid (Figure 26). True to the experimental design, the rumen colloid and supernatant had ($P<0.0001$) higher molybdenum in the $Mo^{++}Fe^{+}$ treatment than the other treatments (19±8 mg/kg DM and 31±28 µg/l in $Mo^{++}Fe^{+}$ vs x̄ 2±0.6 mg/kg DM and x̄ 3±1.1 in the other treatments combined). Unfortunately, there was no significant difference between the Mo^{+} treatment and the non-molybdenum treatments (Figure 25b). In general, <15% (x̄ 13±10%) of molybdenum was associated with the rumen supernatant, although, ($P<0.01$) more (19±18%) was associated with the supernatant in the $Mo^{++}Fe^{+}$ treatment (Figure 25c). No effect of time was observed for the percentage solubility.

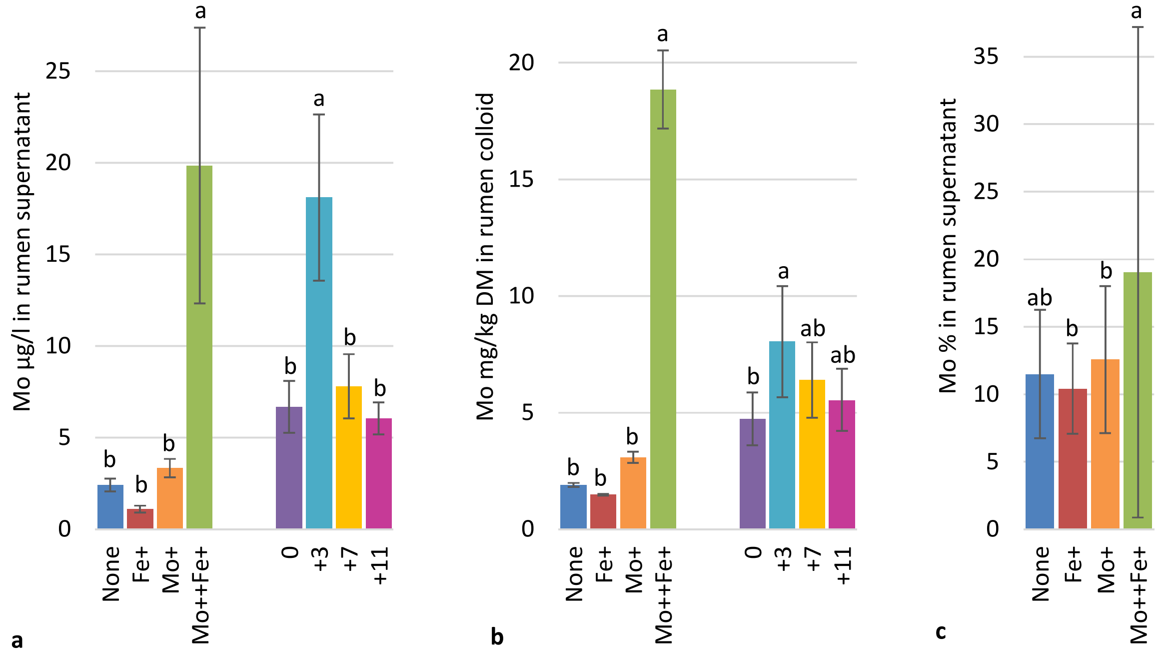

Figure 25: Molybdenum concentration in rumen fluid supernatant (a), colloid (b) by treatment and by time. Percentage of molybdenum in the supernatant fraction (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment or time.

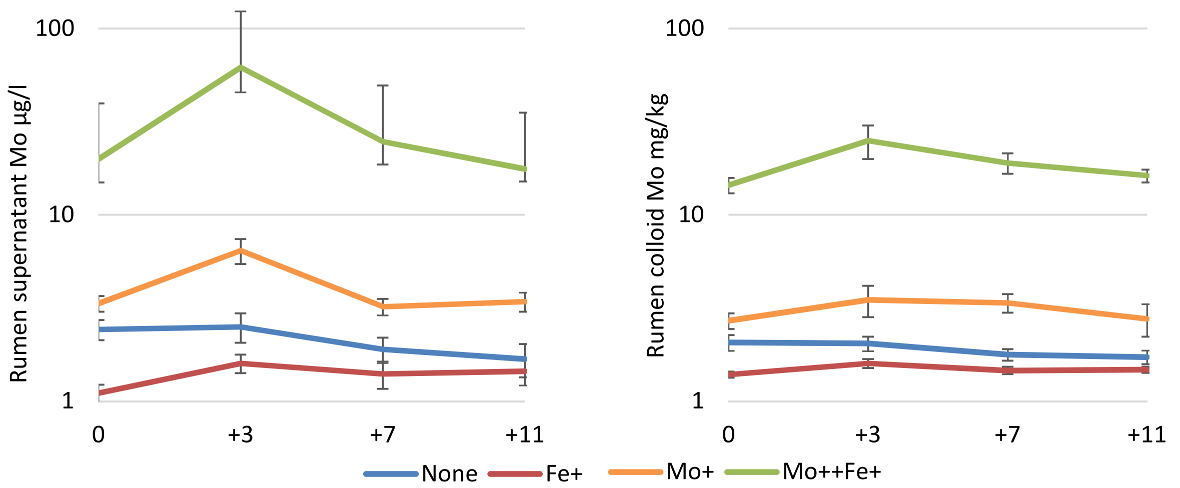

Figure 26: Treatment x time interaction (logarithmic scale) for molybdenum in rumen supernatant (left) and rumen colloid (right). Error bars denote standard error of the mean.

As expected from the experimental design, molybdenum in the omasal colloid (Figure 27b) was ($P<0.0001$) higher (19±4 mg/kg DM) in the $Mo^{++}Fe^{+}$ treatment, followed by the Mo^{+} and non-antagonist treatment (3±0.6 mg/kg DM), with Fe^{+} containing the least ($P<0.0001$) molybdenum (2±0.2 mg/kg DM). However, in the supernatant (Figure 27a), only the molybdenum in the $Mo^{++}Fe^{+}$ treatment was ($P<0.0001$) higher than all the other treatments (14±19 µg/l). The high molybdenum (Mo^{+}) treatment had the next highest molybdenum content (3±3 µg/l), followed by the high iron (Fe^{+}) treatment (2±1 µg/l) and non-antagonist treatment (2±1.6 µg/l), but these did not differ significantly to each other. Interestingly, a ($P<0.0001$) greater percentage of molybdenum was associated with the supernatant in the omasal-flow fluid (~90%) than the rumen fluid (Figure 27c). A ($P<0.01$) lower percentage of molybdenum was found in this fraction in the Mo^{+} treatment (89±9%) than the other treatments ($\bar{x}$ 93±6%, Fe^{+}, $Mo^{++}Fe^{+}$ & none). In the opposite trend to iron, molybdenum did not appear to accumulate in the omasal-flow. Less ($P<0.01$) molybdenum (~3 times) was associated with the omasal-flow supernatant and no difference was found for the colloidal fraction between the two regions.

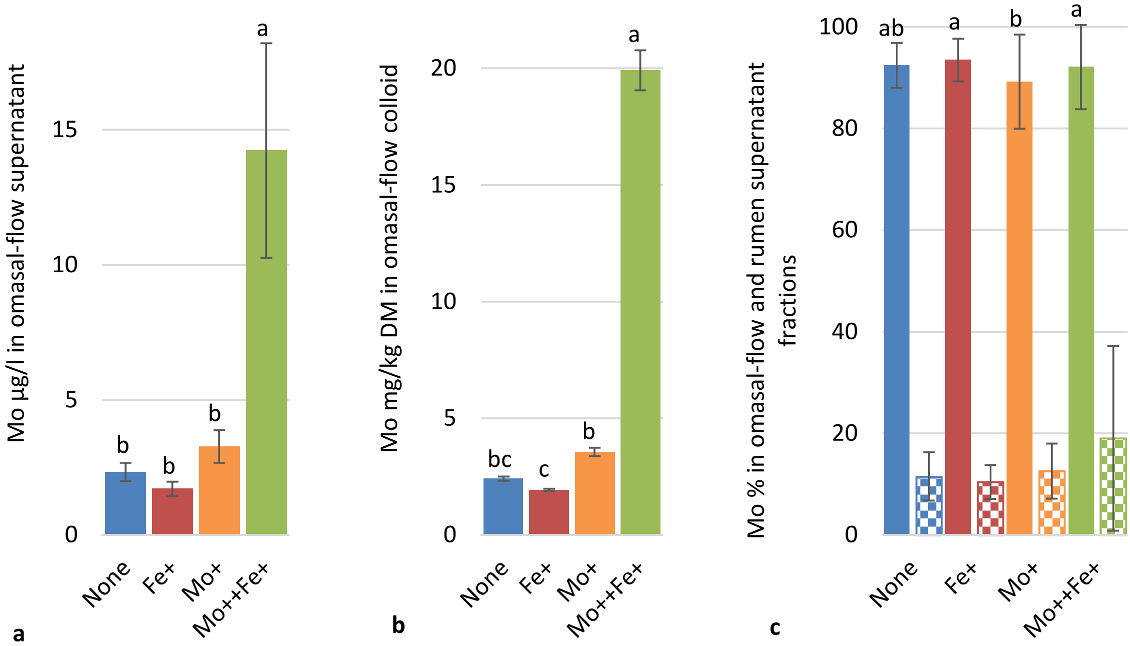

Figure 27: Molybdenum concentration in omasal-flow supernatant (a) and colloid (b) by treatment. Percentage of molybdenum in the supernatant fraction of omasal-flow (solid colour) and rumen fluid (checked colour) (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment. Significant differences for % Mo in rumen fractions previously reported in Figure 25c.

Concentration of molybdenum from faeces was in line with the treatment concentrations. Where molybdenum was not added ($P<0.0001$) less was measured. The $Mo^{+}$$^{+}Fe^{+}$ treatment which contained the most molybdenum measured the most ($P<0.0001$), followed by Mo^{+} ($P<0.0001$). However when lixiviated, the opposite trend appeared. Less ($P<0.0001$) molybdenum was measured from the treatments which contained elevated molybdenum than the ones which had no added molybdenum ($\bar{x}$ 5±1% Mo^{+} & $Mo^{++}Fe^{+}$ vs $\bar{x}$ 3±0.9% none & Fe^{+}).

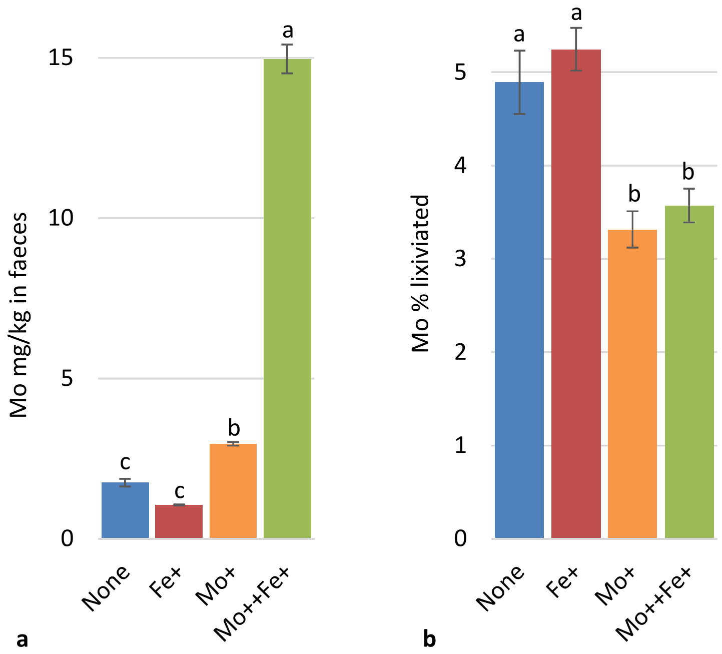

Figure 28: Molybdenum concentration in faeces (a) by treatment. Percentage of molybdenum lixiviated (b) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment.

The only (P<0.05) change in sulphur concentration was found in the omasal-flow supernatant. In this fraction, the sulphur content was higher in the non-antagonist treatment (20±6 mg/l) than the $Mo^{++}Fe^{+}$ treatment (17±5 mg/l) (data not shown).

5.4.2 Tracing copper through digestion

Across all treatments, both colloidal and supernatant fractions of rumen fluid had an (P<0.001) increase in copper concentration at 3 h post-feeding; reflecting the presence of the treatment dose in the rumen contents (Figure 29). Before showing a pattern returning towards basal state at the later sample points. No treatment x time interaction was reported. The majority of the copper in the rumen fluid was associated with the colloidal fraction (P<0.001). Only ~20% ($\bar{x}$ 18±13%) of copper from rumen fluid was held in the supernatant. This was consistent across all time points and treatments.

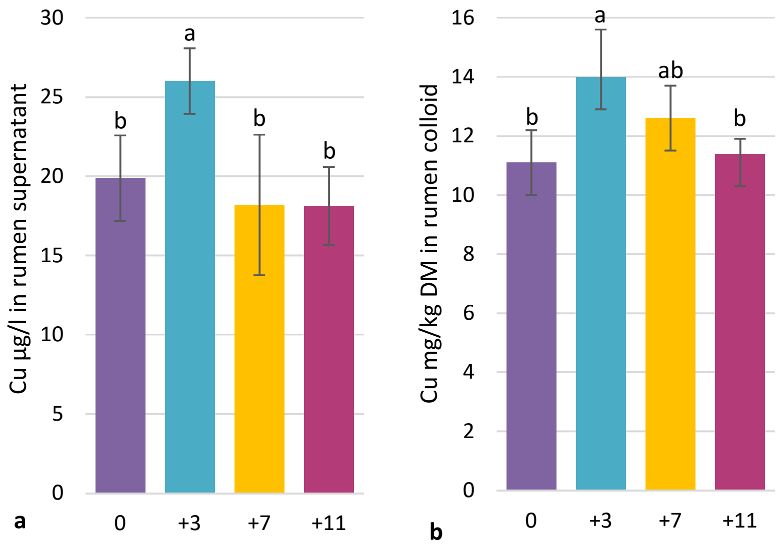

Figure 29: Copper concentration in rumen fluid supernatant (a) and colloid (b) by time. Error bars denote standard error of the mean. Different letters denote significant difference by time.

Copper concentration in the omasal-flow supernatant and colloid were not affected by time point. However, the concentration of copper in the colloidal fraction was typically much higher (P<0.01) than the other parameters sampled, compared in Table 8. Measurements ~47 times higher (P<0.0001) than the rumen or faecal samples were found in the omasal-flow fluid, although, this parameter had a high degree of variability and a high standard error. In general, slightly less copper (x̄ 16±9%) was associated with the supernatant in the omasal-flow fluid in comparison to the rumen fluid, but this difference was not significant.

Table 8: Comparison of copper concentration at different regions of the digestive tract. Absolute concentration values expressed as mean concentration across all treatments ± standard deviation and as a percentage concentration relative to rumen fluid concentration.

	Copper concentration in liquid fraction (supernatant) µg/l	**Copper concentration in colloidal fraction and faeces mg/kg DM**	**Relative concentration %**
Rumen fluid	20.6 (±11.3)	12.2 (±3)	**100**
Omasal-flow	934 (±1,732)	611.9 (±796.2)	**4,713**
Faeces	-	14.8 (±2.2)	**45**

No significant difference was found in faecal copper concentration between time points. The mean faecal copper concentration without antagonists present (none) was 14.5 (±2) mg/kg DM with little deviation across sample points.

5.4.3 The effect of antagonists on copper movement

In the rumen fluid, no significant differences were found between the treatments for copper content of the rumen colloid (Figure 30b). The treatments followed the pattern from most to least; high antagonist (Mo^{+}+Fe^{+}), high iron (Fe^{+}), non-antagonist (none) and high molybdenum (Mo^{+}) over a small range of 12-11 mg/kg DM. In the rumen supernatant (Figure 30a), the most ($P<0.0001$) copper was associated with the supernatant in the Fe^{+} treatment (31±8 µg/l), followed by the Mo^{+}+Fe^{+} treatment (24±12 µg/l) which was also ($P<0.0001$) more than the other treatments ($\bar{x}$ 14±6 µg/l, none and Mo^{+} combined). There were no significant differences in the supernatant percentage across the treatments (Figure 30c). No treatment x time interactions were found for any of the copper parameters.

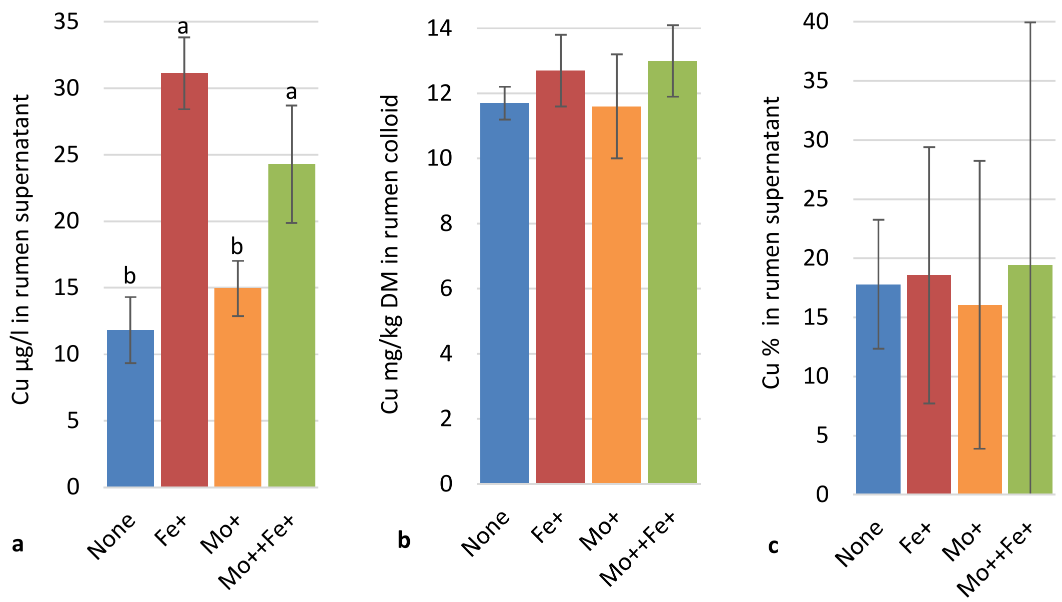

Figure 30: Copper concentration in rumen fluid supernatant (a) and colloid (b) by treatment. Percentage of copper in the supernatant fraction (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment.

In the omasal-flow, both colloid and supernatant, the effect of treatment appeared to be masked by high variability (Figure 31). The mean copper reported for the non-antagonist treatment was higher in the omasal-flow supernatant (1,124±2,510 µg/l) than where antagonists were present ($\bar{x}$ 871±1,397 µg/l, $Mo^{++}Fe^{+}$, Fe^{+} & Mo^{+}), although, none of the differences were significant (Figure 31a). In the omasal colloid (Figure 31b), the most copper was present in the $Mo^{++}Fe^{+}$ treatment (922±1,089 mg/kg DM), followed by Mo^{+} (676±833 mg/kg DM), non-antagonist (467±489 mg/kg DM) and lastly Fe^{+} (383±565 mg/kg DM), but, these were not significant. A ($P<0.05$) higher percentage of the total copper was in the supernatant of the $Mo^{++}Fe^{+}$ treatment (20±11%) than the Fe^{+} treatment (12±7%). Despite the large accumulation between the rumen and omasal-flow, there was no significant difference between the percentage of copper in the supernatant fractions of the two regions (18±13 and 16±9 % rumen and omasal-flow respectively), and there was no significant effect of treatment observed (Figure 31c).

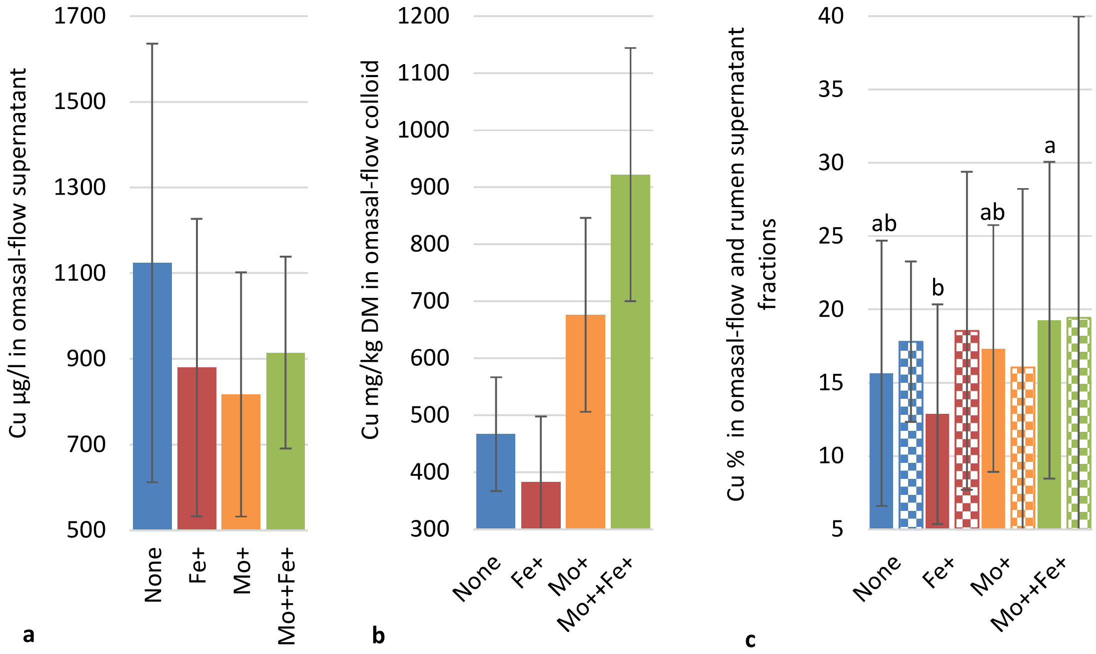

Figure 31: Copper concentration in omasal-flow supernatant (a) and colloid (b) by treatment. Percentage of copper in the supernatant fraction of omasal-flow (solid colour) and rumen fluid (checked colour) (c) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment.

In the faeces, only the Mo^+ treatment (P<0.01) increased faecal copper concentration to 16 (±2) mg/kg DM. Although, the $Mo^{+}{}^{+}Fe^+$ treatment had numerically higher faecal copper concentration to 15 (±2) mg/kg DM, this was not significant. Interestingly, the lowest faecal copper concentration was from the Fe^+ treatment (14±2 mg/kg DM); which was (P<0.01) less than the Mo^+ treatment. More (P<0.0001) copper was lixiviated from faeces in Fe^+ (24±17%) and none (16±7%) treatments while $Mo^{++}Fe^+$ (8±6%) lixiviated the least copper (P<0.0001)(Figure 32).

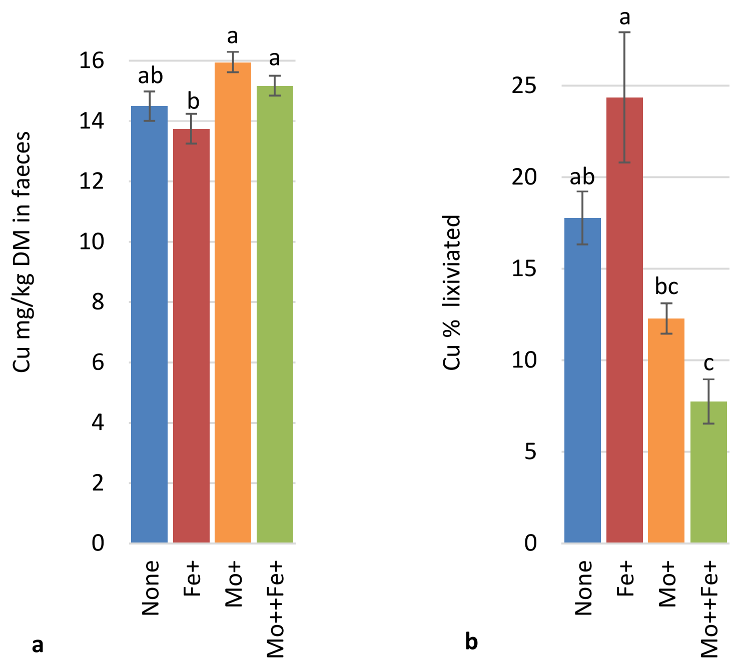

Figure 32: Copper concentration in faeces (a) by treatment. Percentage of copper lixiviated (b) by treatment. Error bars denote standard error of the mean. Different letters denote significant difference by treatment.

5.4.4 The effect of experimental period

Throughout the trial an effect of period was observed. The effect was highly variable, affecting some parameters ($P<0.0001$) but not others. The effect was not consistent for treatment or measured parameter which suggests it may have been due to external environmental influences. The parameters which reported a significant effect of treatment showed either period 2 or 3 yielding higher values. As these were the middle periods and period 1 did not differ to period 4 this supports the hypothesis that these differences were external to the trial and were not the influence of treatment.

5.4.5 Copper absorption and systemic thiomolybdate detection

None of the blood parameters measured during the trial were significantly affected by time and no treatment x time interactions were reported. Using the reference ranges reported in Telfer *et al*., (2004) mean values were at the bottom end of marginal (low) range ($\bar{x}$ 2,015±83 U/g Hb). High molybdenum (Mo^{+}) had the highest SOD value, which was ($P<0.01$) higher than $Mo^{++}Fe^{+}$ and non-molybdenum treatments (Figure 33a). Interestingly, SOD for the non-antagonist treatment was the lowest in this data and was the only treatment which was in the deficient range (1,985±78 U/g Hb) (data not shown). Serum caeruloplasmin activity was not affected by treatment and remained within the lower end of normal range (16.65±6.7 mg/dl) throughout the trial (Telfer *et al.*, 2004) (data not shown). Plasma copper concentration was affected by treatment, period and time. The $Mo^{++}Fe^{+}$ treatment reported the highest plasma copper concentration (13.2±1.6 µmol/l), although all treatments reported plasma copper concentration within normal range (Telfer *et al.*, 2004). The plasma copper from the high antagonist treatment was ($P<0.05$) higher than the Fe^{+} treatment which reported the lowest plasma copper concentration (12.4±1.5 µmol/l), shown in Figure 33a. Using caeruloplasmin to plasma copper ratio (Cp:Cu) all the animals were in the low and marginal-low ranges (range 0.9±0.4-1.3±0.3), no difference between treatments was detected (data not shown).

Plasma molybdenum was also affected by period and treatment. The $Mo^{++}Fe^{+}$ treatment had ($P<0.0001$) higher plasma molybdenum (1.6±0.5 µmol/l), than the non-antagonist treatment (0.2±0.1 µmol/l), which was ($P<0.0001$) higher than the lowest plasma molybdenum values which were reported in the Mo^{+} and Fe^{+} treatments which did not differ significantly to each other ($\bar{x}$ 0.1±0.03 µmol/l), shown in Figure 33b. Throughout the trial, TCA insoluble copper concentrations were variable, some individual values were well above the threshold for thiomolybdate formation (>1). However, means across the trial were negative for thiomolybdate formation (<0. range -1.1 to -1.6 µmol/l) (data not shown).

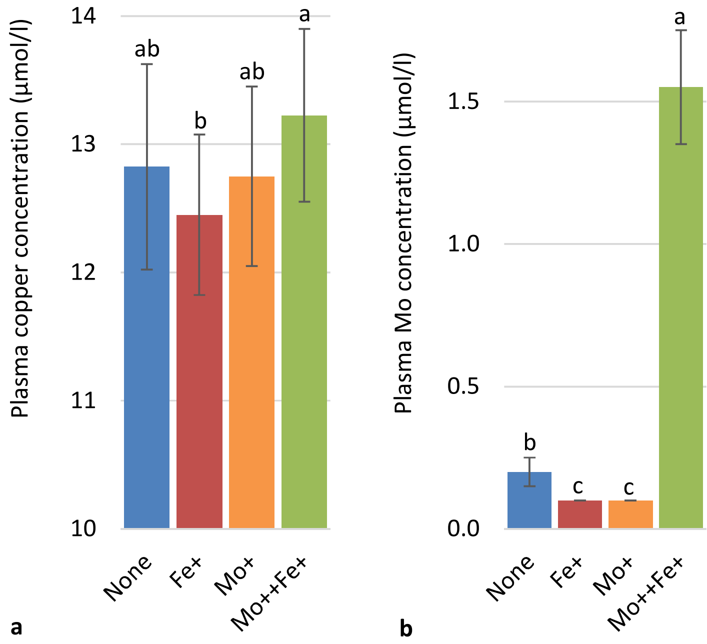

Figure 33: Variation by treatment for plasma copper concentration (a) and plasma molybdenum concentration (b). Error bars denote standard error of the mean. Significant effects of treatment denoted by different letters.

5.5 Discussion

5.5.1 Tracing copper and its antagonists through digestion

The rumen fluid colloidal and supernatant fractions demonstrated an initial peak in copper concentration in line with treatment administration followed by a decline through the later sample points returning to basal by the following day. These results suggest that dietary copper is retained in the rumen for between 3-7 h post feeding before moving further through the digestive tract; in accordance with the findings of Caldera *et al.*, (2019). The majority of copper appeared to be associated with the colloidal fraction of the rumen fluid in this work (~80%). Although the rumen mat was not sampled in this trial, the rumen colloidal fraction measured represents the fine particulate of the solid phase suspended within the liquid phase. The association of copper in the solid phase colloidal fraction has also been noted by others where both thiomolybdate is present and where copper is in an available form (Price and Chesters, 1985; Price *et al.*, 1987).

The pattern of copper association in the rumen supernatant fraction was unexpected. Significantly higher copper was measured in the Fe^{+} treatment which was higher than the $Mo^{++}Fe^{+}$ treatment, both of which were significantly higher than the remaining treatments. The association of copper in the rumen supernatant appeared linked to the presence of iron. Although, the error resulting in the $Mo^{++}Fe^{+}$ treatment containing a 10-fold increase in molybdenum concentration than the Mo^{+} treatment may have distorted this effect. Where molybdenum alone was present, no increase in rumen supernatant copper was observed. The findings here suggest that the iron-sulphur pathway may have an effect within the liquid phase; potentially forming a soluble compound which is too large for absorption. This would also explain the higher concentrations of iron and copper in the Fe^{+} faeces, of which, a significant proportion of both iron and copper could be dissolved through lixiviation; suggesting formation of a complex which may be water soluble but was not absorbed during digestion. It was also observed that the percentage of total iron which appeared in the supernatant was higher where iron had not been added through treatment. This suggests that elevated iron can saturate the liquid phase and associate with the colloidal fraction.

Although not significantly different, the highest concentration of colloidal-bound copper was found in the $Mo^{++}Fe^{+}$ treatment; consistent with a proportion of copper associating with molybdenum as thiomolybdate in this fraction (Gould and Kendall, 2011). Additionally, this treatment reported a higher percentage of copper in the supernatant; perhaps linked to the impact of iron. The percentage of molybdenum in the rumen fluid supernatant for the $Mo^{++}Fe^{+}$ treatment was also higher and was a higher dose than the Mo^{+}, providing support for the notion that thiomolybdate may saturate within the solid phase and be found within the liquid phase (Price *et al.*, 1987). It was interesting to note, that the Mo^{+} treatment appeared to retain the least copper in the colloidal fraction. Unfortunately, due to the 10-fold increase of molybdenum in the $Mo^{++}Fe^{+}$ treatment compared to the Mo^{+} treatment comparisons between the two treatments could not be directly made. In explanation of the lack of significant difference in colloidal-bound copper where molybdenum is present, and to explain the lack of colloidal copper retention where molybdenum alone was added it is possible that a proportion of insoluble copper associated with the solid phase of the rumen contents, sufficiently precipitated that it was not represented in either the liquid or colloidal samples collected. Thus, suggesting that for the Mo^{+} treatment in particular, there may have been formation of copper-thiomolybdate that precipitated out of the rumen fluid. This was further supported by the only significant change in sulphur concentration. Where the concentration of sulphur in the $Mo^{++}Fe^{+}$ treatment was lower than the non-antagonist; supporting the hypothesis for copper-thiomolybdate precipitation. All this evidence is consistent with a higher concentration of copper reported in faeces for these treatments alongside a lower lixiviated percentages.

The flow of copper from the rumen into the omasal-flow fluid (colloid and supernatant combined) appeared to occur at a steady rate. As the copper travels through into the omasum it also appears to accumulate; especially in the colloidal fraction. Although, this appears to be a highly variable process. This effect has not been previously reported or studied. Others have found that omasum plays an important role in the absorption of macro-minerals; potassium, phosphorus and calcium (Tuori *et al.*, 2006), but its role in trace minerals has not been studied or reported. This finding warrants further investigation to discover the purpose of mineral accumulation in this region. Around twice as much iron was also found to accumulate in the omasal-flow, but this was not the case for molybdenum. Molybdenum in the colloidal fraction and supernatant of the omasal-flow remained similar to the rumen. Unexpectedly, the omasal-flow supernatant was lower than the rumen supernatant, especially for the $Mo^{++}Fe^{+}$ treatment which had around half of the molybdenum concentration of the rumen. These changes in elemental concentration have not previously been documented and cannot yet be fully explained.

Without an antagonist challenge, it can be speculated that copper may begin to solubilise within the omasal-flow supernatant; allowing for its potential absorption, potentially due to a slight alteration of pH. Whereas when an antagonist challenge from molybdenum is present, copper is more likely to associate with the molybdenum in the omasal colloid. This data is consistent with data from previous studies that thiomolybdate associates with particulate and flora in the solid phase of the rumen fluid which acts as a temporary intermediate, which then interacts with the solid phase constituents (Campbell *et al.*, 1974; Allen and Gawthorne, 1987;

Price *et al.*, 1987; Suttle, 1991). Once complexed with copper, thiomolybdates are able to precipitate out of the rumen fluid, and which the current data may now suggest are eventually washed into the omasum where they accumulate.

Changes in antagonist concentrations also appear to occur in this region. Iron appeared to follow the pattern of supplementation in rumen fluid, but in the omasal-flow fluid there was no difference in its distribution for treatment in the colloidal fraction. In the supernatant, a larger proportion of iron was found where both antagonists were present. Suggesting that if thiomolybdate has bound copper into the solid phase iron can retain its solubility in the liquid phase. It was interesting to note that iron concentration in the Fe^{+} treatment was as low as the non-antagonist treatment. Possibly suggesting some iron had precipitated into the non-supernatant suspended solid phase; potentially as a bound complex which may include some copper; explaining copper's loss from both supernatant and colloidal fractions in the Fe^{+} treatment. Theoretically, there may be two modes by which iron can affect copper availability in the rumen and omasum. Firstly, the presence of iron may form an insoluble complex which precipitates out of the colloidal fraction held in the liquid phase. Secondly, it may draw copper into a soluble non-absorbable complex held within the liquid phase supernatant. Although evidence of a second mode remains largely conjecture.

Faecal copper concentration appeared to occur at a steady rate during the trial. It was interesting to note that highest concentration of copper concentration in faeces was from the Mo^{+} treatment and not the $Mo^{++}Fe^{+}$ treatment. This suggests that despite a large increase in molybdenum and iron concentration more copper was egested from the animal's digestion where molybdenum alone was present. After lixiviation, a higher percentage of copper could be dissolved from the Mo^{+} treatment than the $Mo^{++}Fe^{+}$ treatment; at 12% and 8% respectively. These results were not significantly different to each other but were significantly lower than the other treatment groups. Indicating that, theoretically, insoluble copper-thiomolybdate had formed in both molybdenum treatments which could not be dissolved through lixiviation.

There were no apparent effects of treatment on the concentrations of molybdenum and iron in faeces; which followed a pattern in line with the fed concentrations. However when lixiviated, there was an interesting effect on molybdenum concentration; which reported less soluble molybdenum in treatments where molybdenum had been added, than those where it had not. This suggests that although the molybdenum was egested in line with its intake, a large proportion of the molybdenum had become insoluble during digestion; supporting the hypothesis of copper binding, forming copper-thiomolybdate.

A summary of the key conclusions on copper movement and hypotheses for binding through digestion are shown in Figure 34.

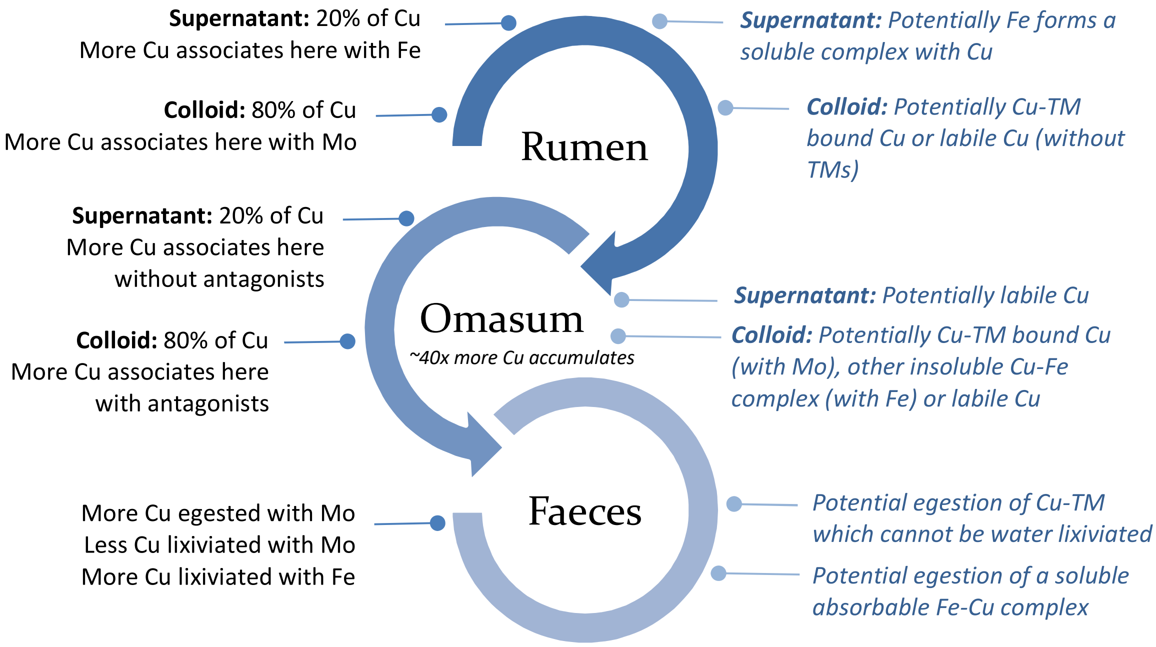

Figure 34: Movement of copper through digestion and influence of antagonists. Conclusions from data are shown on left side, hypotheses for binding are shown on right side in blue italic text.

5.5.2 Copper absorption and thiomolybdate detection

The trial was designed to investigate the short-term effects of antagonist action on digestion and absorption. SOD activity was measured as a medium-term copper status indicator and was not expected to be notably altered by treatment. However, the data did report significantly higher SOD activity in the Mo^{+} treatment. The values remained at the bottom of marginal (low) range (marginal range: 2,000-3,000 U/g Hb) for the antagonist containing treatments it was interesting to note that the non-antagonist treatment was significantly lower than the high molybdenum treatment and this was the only treatment to report values into the deficient range (Telfer *et al.*, 2004). This may be explained, as SOD activity has also been reported to decline with age in cattle (Giergiel and Kankofer, 2014). While the differences found in the present trial may be due to treatment effects it is likely that the overall SOD activity was affected more by the age of the animals than the direct effects of an antagonist containing diet.

Plasma copper concentrations throughout the trial remained within normal range (12-19 µmol/l, normal range: 9.4-20 µmol/l). The difference reported between the $Mo^{++}Fe^{+}$ and Fe^{+} treatments may be linked to a falsely elevated plasma copper status from systemic thiomolybdate sequestering biological copper in the $Mo^{++}Fe^{+}$ treatment and an increased digestive binding preventing systemic absorption in the Fe^{+} treatment. However, this parameter was also subject to vast variation between each of the animals, potentially linked to individual differences in initial copper status and genetic copper tolerance. Time was also found to have an ($P<0.05$) effect on plasma copper status, with plasma copper steadily rising from 0 to 7 h post-treatment. Suggesting a linear absorption of copper from digestion. Caeruloplasmin activity to plasma copper ratios (Cp:Cu) are used to indicate the difference between a thiomolybdate challenge and an iron challenge (Telfer *et al.*, 2004). Animals with Cp:Cu values less than 1.5 are anticipated to be indicative of loss of caeruloplasmin activity from an interaction with thiomolybdate where plasma copper concentrations are within normal range, such as those reported in the present work. The current data expressed mean values for each of the treatments below this threshold, which did not differ significantly to each other, but supported the digestive hypothesis of thiomolybdate formation. The reported TCA insoluble copper concentration data had no evidence of thiomolybdate formation; with all treatments showing no significant difference and returning mean values which did not support the hypothesis for thiomolybdate formation. The lack of TCA evidence where systemic thiomolybdates are anticipated is not unique to this study. Other researchers have found, particularly in cattle, that under conditions which were anticipated to produce thiomolybdate formation, the subsequent TCA insoluble copper fraction failed to form consistently in comparison to controls, despite other copper status markers demonstrating the expected effects (Bremner and Young, 1978; Raisbeck *et al.*, 2006). One study suggested this may be an effect of an undefined physiological factor in this species (Raisbeck *et al.*, 2006). The lack of an effect on TCA insoluble copper concentration in cattle where it can be found in sheep (Gould and Kendall, 2011), has also been observed in the samples submitted to the Nottingham University Veterinary Nutritional Analysis (NUVetNA) service, which led to the cessation of this analysis type for commercial purposes (N. R Kendall 2019, personal communication, 15 July).

Elevated plasma molybdenum concentrations were reported in the $Mo^{++}Fe^{+}$ treatment; far in excess of all the other treatments. This represented an elevated absorption of molybdenum in line with this treatment's elevated concentration; which was larger than originally intended due to the calculation error. Where plasma molybdenum is observed it is not possible to determine using the methods in this trial whether molybdate, rather than thiomolybdate was absorbed. Molybdate is highly absorbable (discussed in Section 1.7.1), if the molybdenum content of the treatment exceeds the binding capacity of available sulphide it is likely that molybdate would be present in the blood. However, the rumen molybdenum concentrations in this work were calculated at 0.03 mg/l (300 nM) in the $Mo^{++}Fe^{+}$ treatment and 0.003 mg/l (30 nM) in the Mo^{+} treatment. Both of which should provide a S^{-}:Mo ratio >10:1; (assuming rumen sulphide conversion in line with Laurie, 2000). These concentrations should be more than sufficient to promote thiomolybdate, as tetra-thiomolybdate, formation *in vivo* without saturation of sulphide (Clarke and Laurie, 1980), although this cannot be conclusively established in the present work.

It was interesting that the second highest plasma molybdenum concentration was reported in the non-antagonist treatment not the Mo^{+} treatment; which reported one of the lowest plasma molybdenum concentrations. Thus, it may be that a proportion molybdate was absorbed in the $Mo^{++}Fe^{+}$ treatment and that the comparably lower concentration in the Mo^{+} treatment represents copper-thiomolybdate binding in digestion without thiomolybdate or molybdate absorption.

The lack of significant differences in blood parameters in this trial may be linked to the limited number of animals used in the design. Individual variation in combination with a small number of animals suggests that any potential effects of treatment were masked by this. Limitations placed on the trial through the use of fistulated animals and tie-stall cubicles prevented larger animal numbers from being used. To gain a greater insight into the changes in blood parameters associated with the treatments, rather than individual variation, would require a greater number of animals and therefore a different trial design focussing on faecal concentrations and blood parameters over rumen and omasal fluid concentrations. Overall, the validity of this trial was weakened due to the removal of one of the animals during the trial. Serious declines in dietary dry matter intake for this animal over a period of several days meant that the treatment doses were no longer accurate and a reduced digestive motility was unlikely to yield reliable results. The animal was assessed for ill health and none was found. However, the animal was withdrawn from the final trial period and this animal's data was discarded from the entire trial. Trial data was subsequently analysed only for the remaining 3 animals. This has added to individual variation found in the remaining animals which has subsequently impacted on the reliability of the conclusions. The loss of this animal also weakened the statistical power of the Latin square design. Unfortunately, this was not the only factor to weaken the Latin square design. The miscalculation of molybdenum content in $Mo^{++}Fe^{+}$ treatment also added further dilution to the statistical power. The notable effects ($P<0.05$) of period throughout the trial in some of the digestive and blood parameters and across the elements studied were possibly linked to the weakened design. Due to practical restrictions, a short acclimation period to each treatment of only 3 days was used in this trial alongside a short rest period of only 2 days. Other short term studies of similar design to this one used longer acclimation periods of 5-7 days (Caldera *et al.*, 2019). The short periods used in this trial may not have been sufficient to produce the full effects of treatment and may not have been sufficient to prevent carry-over between the treatments. In future work, a more robust calculation of the treatments alongside longer rest periods between treatments and longer acclimation periods to each new treatment may assist in avoiding these effects.

5.6 Conclusion

The present work has demonstrated that copper is cleared from the rumen from ~7 h of feeding. Uniquely, it has been found that the omasal-flow may act as a copper sink; where copper which is passed through from the rumen accumulates across both the supernatant and colloidal fractions in concentrations much higher than that of the rumen or resulting faecal product. Another new finding, was the potential influence of elevated iron sequestering copper into the supernatant of the rumen fluid and the use of lixiviation suggested that a soluble compound too large for absorption may form in this pathway. The use of lixiviation helped differentiate copper which was potentially unavailable and insoluble, presumed to be copper-thiomolybdate, in the molybdenum containing treatments over the concentrations of copper which could still be solubilised in water.

6.0 XAS modelling of liver copper compounds

6.1 Introduction

It has been previously discussed that the complexities of copper provision have contributed to a culture of copper over-supply in some areas of agricultural practice (Section 1.6.1 and 2.5). Ongoing over-provision leads to 'copper-loaded' animals with the potential for haemolytic crisis (discussed in Section 1.2.2) (Sivertsen and Løvberg, 2014). In this chapter, the impact of copper-loading on the liver of ruminants will be studied.

6.1.1 The role of the liver in copper control

The liver is the major organ associated with copper accumulation and metabolism. It is directly affected by copper intake, and supplementation can have a marked increase on hepatic copper concentration (Dias *et al.*, 2013).

Copper absorbed by the enterocytes in excess of their cellular requirement is trafficked via the blood to the liver, where it is absorbed into the hepatocytes. The reactive nature of copper requires it to be chaperoned to prevent cellular damage (discussed in Section 1.1) (Clarkson *et al.*, 2019). Once inside the cell, chaperone proteins bind to the copper and transport it within the cell to other specific proteins or incorporate it into cuproenzymes (Failla, 1999; Linder *et al.*, 2003; Maryon *et al.*, 2013; Spears, 2013). Advances in our understanding of these pathways are reviewed in Clarkson *et al.*, (2019). The major pathway in the liver processes copper via the Golgi and is known as the secretory pathway. This pathway directs the majority of copper to be incorporated into caeruloplasmin, as holo-caeruloplasmin, which is then effluxed into circulation for distribution to the peripheral tissues (Failla, 1999; De Bie *et al.*, 2005; La Fontaine and Mercer, 2007; Prohaska, 2008; Suttle, 2012). When caeruloplasmin-bound copper from the peripheral tissues returns to the liver the whole molecule of holo-caeruloplasmin is absorbed for destruction and excretion through the biliary route into the animal's faeces (Linder *et al.*, 2003; Nevitt *et al.*, 2012; Dias *et al.*, 2013).

The Golgi is also responsible for binding copper to metallothionein which can then be stored in the lysosome, here, it acts as an intracellular copper buffer (Dameron and Harrison, 1998; Failla, 1999; Thiele, 2003; Nevitt *et al.*, 2012).

Metallothionein is a cytosolic, cysteine-rich polypeptide that can bind up to 12 Cu^{+} atoms through strong thiolate bonds (Arredondo *et al.*, 2004). Under copper-limiting conditions, copper bound to metallothionein is mobilised using the acidic pH of the lysosome to partially degrade the metallothionein and release its copper into the cytosol (Klaassen *et al.*, 1994; Suzuki *et al.*, 2002; Nevitt *et al.*, 2012). The released copper is delivered, likely by glutathione, in equal supply to the protein and enzyme producing copper chaperones, but not into the secretory pathway (Vulpe and Packman, 1995; Suzuki *et al.*, 2002; Lutsenko, 2010); thus retaining the copper for intracellular use (Hamza and Gitlin, 2003). This diminishes copper supply to the secretory pathway resulting in the production and secretion of the copper-empty apo-caeruloplasmin, rather than its copper-containing holo form (Suzuki *et al.*, 2002).

Under copper-replete conditions, the copper transporters and proteins are down-regulated (Thiele, 2003; Lutsenko, 2010). Metallothionein expression is increased to bind the excess copper (Hamza and Gitlin, 2003; Nevitt *et al.*, 2012). Copper in excess of the metallothionein capacity is imported into the lysosomal lumen for temporary storage (Polishchuk and Polishchuk, 2016). The increasing intracellular copper concentration induces exocytosis of the lysosome releasing the excess copper into the biliary canal (Petris *et al.*, 1996; De Bie *et al.*, 2005; Collins *et al.*, 2010; Lutsenko, 2010; Polishchuk *et al.*, 2014).

6.1.2 Liver copper concentrations

The critical hepatic copper concentration for toxicity cannot be easily defined (Strickland *et al.*, 2019b). There is no definitive critical concentration at which the metallothionein capacity is overwhelmed. An elevated hepatic copper status does not equate to haemolytic crisis, but it does increase the risk of its occurrence (Minervino *et al.*, 2009; Kendall *et al.*, 2015), discussed in Section 1.2.2.

Post-mortem liver copper concentrations >13,801 µmol/kg DM were once considered evidence of copper toxicity (van der Schee *et al.*, 1983). However, sheep considered healthy and showing no clinical signs, have been found at slaughter with liver copper concentrations in excess of 14,000 µmol/kg DM (Menzies *et al.*, 2003; Sivertsen and Løvberg, 2014). In line with supplemental attitudes of sheep farmers (discussed in Section 2.5), in the UK most finishing lambs were found to have liver copper in the 'normal' (1,405-5,618 µmol/kg DM) range, and were more likely to be below this range than above it (Clarkson *et al.*, 2017). In contrast, across Canada mean liver copper concentration of cull sheep and lambs was 9,444 µmol/kg DM with 6% in excess of 13,800 µmol/kg DM, and in Norway 13% of apparently healthy cull sheep and lambs had liver copper in excess of 8,450 µmol/kg DM with some livers up to 38,869 µmol/kg DM (Sivertsen and Løvberg, 2014).

However, similar to the findings in Chapter 2 which assessed copper supplementation strategies, a recent study found only 34% of UK cull dairy cattle fell within the same 'normal' range and only 8% below 'normal', while 57% exceeded 'normal', with 38% of these in excess of 8,000 µmol/kg DM; the top of the Animal Health Veterinary Laboratories Agency (AHVLA) reference range (Kendall *et al.*, 2015). In support of this, other work suggests as many as 89% of Holstein-Friesian calves fed to the previous legal limit (40 mg/kg DM) may have accumulated liver copper concentrations in the region of 8,450 µmol/kg DM (Miranda *et al.*, 2010). In beef cattle, there was less UK sample data, but 22% reported as 'normal', 55% below 'normal' and 23% above. Of these, only 3% exceeded the AHVLA reference range (Kendall *et al.*, 2015). However, data from Spain shows liver copper accumulation in beef cattle; with dairy breeds raised as beef accumulating more copper than beef breeds (Galician blonde) of the same husbandry (Pereira *et al.*, 2018). This data suggests that for UK dairy cattle in particular, copper toxicity may present a greater risk than deficiency (Kendall *et al.*, 2015; Strickland *et al.*, 2019b).

If all copper supplementation was withdrawn for animals with high liver copper concentrations it would take around a year of non-supplementation to decline to normal status (Kendall *et al.*, 2015), and normal livers in the region of 3,600 µmol/kg DM would deplete at a rate of 0.57% per day (Grace *et al.*, 2012; Hittmann *et al.*, 2012); taking a further year to reach marginally deficient status (<400 µmol/kg DM) (Grace *et al.*, 2012; Kendall *et al.*, 2015).

6.1.3 Biliary excretion of copper

The liver exerts homeostatic control of copper through biliary excretion, which constitutes the only excretory route for endogenous copper. This homeostatic control acts primarily through regulating the secretion of copper into bile (Harris, 2000; Linder *et al.*, 2003; Stern *et al.*, 2007; Collins *et al.*, 2010). Biliary excretion allows both the enterohepatic recycling of copper and the excretion of excesses. According to the kinetic modelling of Buckley, (1991), ~60% of the total copper excreted in the faeces in non-pregnant cattle was from bile (Gooneratne *et al.*, 2013).

When hepatocytes are exposed to increasing copper concentrations the copper transporters and proteins responsible for uptake into the cell are down-regulated, while metallothionein transcription is up-regulated, via the process described in Section 6.1.1 (described in detail in Clarkson *et al.*, 2019). Under copper-limiting conditions the movement of copper into the secretory pathway is diminished (Stern *et al.*, 2007; Lutsenko, 2010), and copper bound to metallothionein is released (as previously described in Section 6.1.1). When the liver reserves become depleted or cannot be mobilised fast enough to meet the deficit in supply, caeruloplasmin synthesis decreases and plasma copper falls below normal (Suttle, 2010).

It has been demonstrated that biliary copper excretion in cattle can be increased through feeding diets high in copper, molybdenum and sulphur (Gooneratne *et al.*, 1994). It has been suggested that copper is removed from the lysosome of the hepatocyte into the bile via a direct pathway. This effect was suggested in trials which used both intravenous and intra-duodenal administration of thiomolybdate (Gooneratne *et al.*, 1989b; Gooneratne, 2012). In sheep too, experimental evidence has indicated that feeding sodium molybdate promotes biliary copper excretion through the removal of copper from the lysosome and cytosol of the hepatocytes in copper-loaded animals (Hidiroglou *et al.*, 1984; Kumaratilake and Howell, 1989). The observed effect in both species is attributed to the formation of thiomolybdate exerting its high affinity for copper, even where it is held by metallothionein. It has also been suggested that enterohepatic recycling of copper can be impaired by thiomolybdate due to the effect of molybdenum increasing faecal endogenous loss (Smith *et al.*, 1968), and increasing the rate of liver copper depletion in sheep (Woolliams *et al.*, 1983; Freudenberger *et al.*, 1987).

6.1.4 Species differences

The sensitivity of ruminants to hepatic copper accumulation may be linked to a reduced capacity for metallothionein transcription in the liver. The importance of metallothionein in copper handling is demonstrated as metallothionein knock-out animals, even from monogastric species, are hypersensitive to copper (Tapia *et al.*, 2004). Sheep have a limited ability to synthesise metallothionein in response to rising copper concentration, caused by a poor transcription response in the lysosome, and they appear to have a restricted capacity to accumulate copper bound to metallothionein in the liver (Corbett *et al.*, 1978; Saylor and Leach, 1980; Saylor *et al.*, 1980; Bremner and Beattie, 1995; López-Alonso *et al.*, 2005a). In combination with a limited ability to increase biliary copper excretion in response to copper intake (López-Alonso *et al.*, 2005a). These factors result in copper accumulation in the liver which is potentially bound only to temporary chaperones.

Cattle also have a lower capacity to store copper bound to metallothionein and a limited capacity to induce metallothionein in response to copper intake in comparison to monogastric species, but which appears superior to sheep (Saylor and Leach, 1980; Bremner and Beattie, 1995). Furthermore, in cattle and sheep the copper-buffering capacity decreases as hepatic copper loading increases (López-Alonso *et al.*, 2017). Pigs and dogs have around 500-600 mg/kg wet weight (WW) metallothionein in their liver, while sheep and cattle have only ~200 mg/kg WW (Henry *et al.*, 1994; López-Alonso *et al.*, 2005b). Metallothionein transcription appears to reach a plateau of total copper concentration ~1,607 mg/kg DM (25,347 µmol/kg DM) in cattle and ~571-643 mg/kg DM (9,006- 10,142 µmol/kg DM) in sheep (Corbett *et al.*, 1978; Saylor and Leach, 1980; López-Alonso *et al.*, 2005b, 2005a). Theoretically explaining why cattle appear to be more copper tolerant than sheep and why both species appear sensitive in comparison to monogastric species such as pigs.

If the influx of copper exceeds the capacity of the metallothionein and lysosomal uptake, unbound copper will occur in the cytosol and begin to enter the nucleus, causing severe cell damage (López-Alonso *et al.*, 2005b, 2017; Strickland *et al.*, 2019b).

6.1.5 Thiomolybdate chemistry and complexing

Since ruminants are also affected by thiomolybdate production and absorption (discussed in Section 1.7). It is of interest to further investigate the presence or influence of hepatic thiomolybdate and its ability to bind to copper in metallothionein (Ogra *et al.*, 1999). While thiomolybdates are able to cross cell membranes, the mechanism by which this takes place is unknown and as yet it has not been determined if copper bound to thiomolybdate can persist in the liver. In order to investigate this it is important to first understand the nature of thiomolybdate binding and chemistry.

Thiomolybdate forms an incredibly strong compound with copper. Extraordinarily large stability constants have been measured for Cu^{+}-thiomolybdate complexes (Clarke and Helz, 2000; Helz and Erickson, 2011; Helz *et al.*, 2014). Copper-thiomolybdate complexes have general formula $(Cu_{m})_{n}$ $MoS_{4}^{\pm var}$ where $_{m}$=1 or 2 and $_{n}$=1-6 (Zhang *et al.*, 2001; Helz and Erickson, 2011). Oxidation states of the metals in the formed copper-thiomolybdate complex are Cu^{+} with Mo^{6+} or pentavalent Mo^{5+} (Binnie *et al.*, 1970; Laurie, 2000; Helz and Erickson, 2011). Within thiomolybdate, the molybdenum centres and both the bridging and terminal sulphur ligands (represented in Figure 35) are able to exist in multiple oxidation states (Quagraine, 2002). Copper appears as Cu^{+} regardless of the oxidation state of the reactant, while sulphur can exist as the anionic complexes; S^{2-}, S_2^{2-} or S_4^{2-}. In unreacted thiomolybdate, molybdenum is present as Mo^{6+}, which can be reduced to lower oxidation states upon reaction into the Cu-(S)-Mo-S clusters (Quagraine, 2002).

As Cu^{2+}, copper has a preference for square planar, distorted square planar, square pyramidal, trigonal pyramidal or axially distorted octahedral formations (Ding *et al.*, 2011; Helsel and Franz, 2015). Monovalent copper (Cu^{+}) has a diamagnetic configuration which allows it to form complexes with flexibility in its geometric arrangement allowing it to form tetrahedral, trigonal or linear arrangements (Ding *et al.*, 2011; Helsel and Franz, 2015). Ligands which impose a tetrahedral arrangement are highly unfavourable for Cu^{2+}, but are suitable for Cu^{+} (Ding *et al.*, 2011).

XRD studies and Electron Paramagnetic Resonance (EPR) spectroscopic studies of the solids formed from tetra-thiomolybdate interactions with Cu^{2+} in aqueous solutions suggests the presence of chains of CuS and MoS_4 tetrahedra connected at their edges (Binnie *et al.*, 1970). In these complexes, each copper is bound to two sulphur atoms that define an edge of a MoS_4^{2-} tetrahedron (Helz and Erickson, 2011). Up to six copper atoms can be bound to one tetrahedron across the tetrahedral faces of the complex, but three or four are most common (Laurie, 2000; Zhang *et al.*, 2009). One or two external ligands are present per copper atom giving the copper atoms three or four co-ordination respectively (Helz and Erickson, 2011). The external ligands are thiols, usually associated with proteins (George *et al.*, 2003; Alvarez *et al.*, 2010). Figure 35 shows the co-ordination geometry for 1-4 copper co-ordination with tetra-thiomolybdate described.

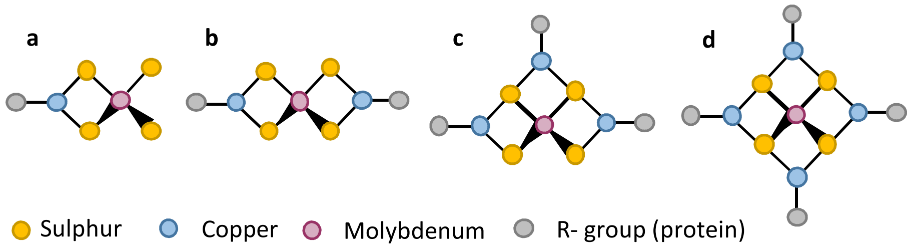

Figure 35: 1 to 4 co-ordination of copper with tetra-thiomolybdate (adapted from George *et al.*, 2003; Zhang *et al.*, 2009). Image a; one co-ordination, b; 2 co-ordination, c; 3 co-ordination and d; 4 co-ordination.

The presence of these compounds, in particular CuS_2MoS_2Cu, a Cu^+ tetra-thiomolybdate, as well as Cu^+ tri, and di-thiomolybdates have been found in rumen digesta (Price *et al.*, 1987). Atomic absorption data consistent with this type of bonding has also been reported in caeruloplasmin and tetra-thiomolybdate treated caeruloplasmin where 6 Cu^+ atoms remained in both complexes, and was suggested to be bound to the thiomolybdate via sulphide groups (Chidambaram *et al.*, 1984).

The stability of an oxidation state within a complex depends on the mode of bonding. Synergistic bonding involving ligand donation and back acceptance conveys an electrical neutrality to the complex providing additional stability to the complex (Rizvi *et al.*, 2015). Due to its lower charge and completely filled *d*-orbitals Cu^+ promotes synergistic bonding and a good degree of back acceptance to the ligand in comparison to Cu^{2+}. This allows Cu^{+} to form more stable complexes with π-acceptor type ligands due to a low degree of back donation from Cu^{2+} by comparison (Rizvi *et al.*, 2015). At around pH 6, representative of a grazing ruminant, the sulphide concentration in the rumen amply exceeds the switch point for conversion of MoO_4^{2-} to MoS_4^{2-} (Erickson and Helz, 2000). Abundant carboxylic acids (~10^{-1} M) produced by fermentation in the rumen could greatly accelerate the rate of conversion through acting as a catalyst for thiomolybdate conversion (Erickson and Helz, 2000). At the point of formation, thiomolybdates are likely to bind to ammonia as ammonium-thiomolybdates, most likely $[NH_4]_2MoS_4$ (Mackenzie *et al.*, 1997a). It is in this complex that they are described as being 'unbound' or 'free' when they are absorbed systemically in ruminants, although there are no studies which confirm this.

Competition studies have shown that copper (Cu^{2+}) complexed to sulphide or EDTA is able to resist displacement by thiomolybdate to a certain extent, but only where the sulphide or EDTA is in excess of thiomolybdate; causing a slower and partial displacement (Quagraine, 2002; Essilfie-Dughan, 2007). If thiomolybdate is in excess or is bound to copper before the addition of the competing ligands even a 1,000-fold excess cannot displace it (Quagraine, 2002).

6.1.6 X-ray absorption Spectroscopy (XAS)

A useful method which can be used to investigate the oxidation state and local co-ordination bonding is X-ray absorption spectroscopy (XAS). X-ray absorption near edge structure (XANES) and X-ray absorption fine structure (XAFS) analyse the spectra obtained through XAS. This analysis is element specific and is sensitive to local bonding (Sarangi, 2010). At characteristic wavelengths, the X-ray absorption of an element changes dramatically; creating an absorption edge (Sarangi, 2010). X-rays are an ionising electromagnetic radiation which provide sufficient energy to excite a core electron within an atom altering its ionisation threshold. Differing core electrons have different binding energies allowing a spectrum of X-ray absorption to be plotted for a specific element (Penner-Hahn, 2004). As the energy from the X-ray radiation scans through the binding energy of the core shell, a sudden increase of absorption appears; corresponding to the absorption of the X-ray photon by a core electron. The excited electron, now a photoelectron, leaves it's position within the shell to occupy a vacant position in an outer shell or leaves the atom entirely for the continuum (Penner-Hahn, 2004). Absorption edges are named according to the shell the core electron originated from. For copper and iron the electron originates from the 1S orbital, termed the K-edge at ~9,000 eV and ~7,000 eV respectively. The energy of the absorption edge and its associated spectra can reveal the identity of the surrounding elements (Sarangi, 2010). The absorption edge is more complex than an abrupt increase. There are weak transitions below the absorption edge termed pre-edge structures and features within 50 eV of the absorption edge in the XANES region. Above the absorption edge, this oscillation is caused by interference between outgoing and back-scattered photoelectron waves and can be studied using XAFS which can extend to over 1,000 eV above the absorption edge. The pre-edge structures can be used to estimate the ligand field, spin state and centro-symmetry. The rising edge can be used to estimate the geometric structure, metal-ligand overlap, ligand arrangement; in some cases, and the charge on the metal centre (Penner-Hahn, 2004). Many edges show significant edge shifts which represent shifts in binding energy alongside changes in oxidation state. The rising edge and edge maxima will shift to a higher energy as the oxidation state increases (Sarangi, 2010). The co-ordination number and geometry effect the metal K-edge shape and energy even in compounds where the metal is present in the same oxidation state (Sarangi, 2010). Where XAFS spectra are gathered for samples comprising a mixture of compounds local co-ordination chemistry can be inferred through the use of principle component analysis (PCA) and linear combination fitting (LCF). LCF is most effective where the spectra of the individual components in the mixture are very different. The sample spectra is compared to reference spectra for all of the compounds suspected to be present. Fitting each of the spectra to the overall spectral shape provides relative proportions of each candidate. PCA models the spectral characteristics of the principal components without a specific reference spectra. Commonly these two methods are used together to try and determine the contributing substances in unknown mixtures. In all XAFS spectra XAFS peak fitting can be used to model individual pathways from candidate atoms using effective scattering factor (FEFF) calculations using the XAFS equation:

$\chi(\kappa) = S_0^2 \sum_i N_i \frac{f_i(k)}{kD_i^2} e^{-\frac{2D_i}{\lambda(k)}} e^{-2k^2\sigma_i^2} \sin\left(2kD_i + \delta_i(k)\right)$. XAFS equation parameters are defined on Page *xxii.* A detailed explanation of the principles which underpin this analysis are explained in Koningsberger *et al.*, (2000).

6.2 Aims & Objectives

The aim of this work was to compare the oxidation state and local co-ordination bonding of copper stored in bovine and ovine livers containing >8,000 µmol/kg DM copper. A secondary aim was to determine if copper was bound to metallothionein and/ or thiomolybdate.

6.3 Materials & methods

6.3.1 Liver preparation

Bovine and ovine livers of high (>8,000 µmol/kg DM) copper status were identified through the use of the NUVetNA database and previous trial work (Clarkson *et al.*, 2017). High liver samples for two different species were selected for comparison in this trial, as opposed to high and low samples from the same species, as samples in the low range would fall below the detection limits of the XAS beamline (<500 µM). Surplus liver, in excess of 300 g which had been continuously stored at -20 ˚C, was prepared through the removal of the outer capsule and slices of ~1 mm thickness were cleaved from the central tissue using a clean scalpel until ~8 g WW had been collected. Livers were immediately returned to -20°C prior to being subject to freeze-drying (Modulyo® M143, Edwards, Leicestershire, UK) for ~100 h. Samples were released from vacuum using nitrogen (PRISM, Air products, Surrey, UK) and split into replicates. One replicate (~0.5 g) was subject to wet acid digestion and ICP-MS using the previously described methods (Sections 5.3.3.2 & 3.3.2.1) to confirm copper concentration. The second replicate was prepared for XAS analysis by grinding into a homogenous powder using a pestle and mortar and sieving to <120 µm in a nitrogen atmosphere. Ground samples were nitrogen flushed, capped and sealed in the nitrogen atmosphere to prevent oxidation. Once at the beamline, powdered samples were prepared into aluminium sample holders (Diamond Light Source, Oxfordshire, UK) by careful placement onto a 7 mm square piece of polyamide film (25 µm thickness, DuPont, Stevenage, UK) and compacted in the centre to align with the sample holder window at a thickness of 0.5-0.8 mm and covered with a second square of polyamide film secured with silicone adhesive polyamide tape (Kapton®, DuPont, Stevenage, UK) to create a sealed compartment before screwing the back on to the sample holder.

6.3.2 Standard preparation

Standard samples for use as QC and fingerprinting of XANES spectra comprised; copper sulphate, cuprous-tetra-thiomolybdate, chalcopyrite and bornite. Copper sulphate was used without further purification ($Cu^{2+}SO_4 \cdot 5H_2O$; Alfa Aesar, Lancashire, UK. >99.9%). Cuprous-tetra-thiomolybdate was prepared through the combination of 0.5 M ammonium-tetra-thiomolybdate ($(NH_4)_2MoS_4$; Sigma Aldrich, Gillingham, UK) and 5 M copper sulphate (as previous) in de-ionised water (Purite hp 160, Suez, Thame, UK. 17 MΩ cm). The solution was inverted and bench vortexed until a colour change to black-brown was observed. At this point the solution was centrifuged at 2,000 *g* for 15 mins (Allegra X-22, Beckman Coulter, High Wycombe, UK). The supernatant was removed using a pipette and discarded. The remaining precipitate was placed on to filter paper (№42, Whatman, Maidstone, UK) to remove any further supernatant and was then left to air dry for 48 h. Chalcopyrite ($Cu^{+}Fe^{3+}S_2^{2-}$) and bornite ($Cu_5Fe^{2+}S_4$) from crystalline ore were coarsely crushed with a hammer and ground into a fine powder and sieved to ensure particle size <250 µm, before analysis using X-ray diffraction (XRD) (Equinox 3000, Thermo Scientific, Altrincham, UK) to confirm the purity and identity of the compounds. Each standard was prepared into a 13 mm diameter pellet using a pelleting press (Atlas 25T, Specac, Kent, UK) containing 120 mg of reference compound (calculated by formula weight) weighed and mixed into homogeneity with dried cellulose binder in an agate mortar. All standards were analysed in transmission mode alongside a copper foil (I20, Diamond Light Source, Oxfordshire, UK) which was placed in-line with the incident beam with all standards.

6.3.3 XAS Analysis

XAS data for the samples was collected in fluorescence mode at beamline I20-Scanning at the Diamond Light Source (Oxfordshire, UK). Samples were subject to the X-ray beam monochromated by Si (111) crystals. The XAS spectra was collected at the copper (8,979 eV) K-edge. Spectra were recorded in fluorescence mode using the 64-pixel Ge detector (Canberra, Oxfordshire, UK) at 90° to the incident beam. The incident and transmitted X-ray intensities were monitored using ionization chambers. During data collection, samples were maintained at a temperature of -196.15 ˚C using an LN2 cryostat (Oxford instruments, Abingdon, UK) to minimise the effects of radiation damage. For each sample, six to eight 30 min scans for copper K-edge data were performed due to the dilute nature of the samples. Radiation damage of the sample was observed when repeated scans were taken at the same point, therefore the beam position was adjusted between each scan. The K-range of the XAS spectra was limited to 10 Å due to the presence of a zinc absorption edge at 9,659 eV, which distorted the XAS signal beyond this point.

6.3.4 Data analysis

All XAS scans, transmission and fluorescence, were calibrated, aligned, background removed, rebinned and normalised using Athena v 0.9.25 (Ravel and Newville, 2005) generating XANES spectra. Where appropriate, scans were merged to reduce the signal to noise ratio at further Å. XAFS spectra were extracted from μ(E) based on an estimate of the free-atom absorption. Data was extrapolated into K-space where the amplitude and phases were calculated using FEFF to fit χ(k) to a variation of non-linear least-squares curve fitting. Historically, this data type is difficult to evaluate in K-space thus, the data and the fit were transformed through Fourier transform to a radial structure function (R-space) for analysis. XAFS spectra were then peak fitted in R-space using IFFEFIT of Fourier transformed data analysed through Artemis v 0.9.25 (Ravel and Newville, 2005). Goodness of fit was determined where R values were <0.02 and Debye–Waller factors (σ^2) were ~0.02.

6.4 Results

6.4.1 XANES

Analysis of XANES spectra from XAS data is an interpretive process. Results are extrapolated from the features of the spectra generated in comparison to previous work, crystallographic data and using fingerprinting techniques.

Analysis of the absorption energy (E^0) indicates that the average oxidation state of copper in both ovine and bovine livers lies between Cu^0 and Cu^+. Cu^+ has typical edge positions which range from 8,983-8,984 eV (Ceko *et al.*, 2014). Whereas the E^0 for Cu^0 is 8,979.3 eV (Thompson *et al.*, 2009). The absence of a feature at 8,979.5 eV indicative of the 1s f 3d transition in Cu^{2+} also supports that copper is present in both species livers as Cu^+ (Zhang *et al.*, 2009). A small degree of reduction was observed in the beam during the experiment which was minimised by moving the sample position before each scan. However, a small proportion of copper reduction may have resulted from photo-reduction. The E^0 for hepatic copper was reported at 8,981 eV specifically, 8,980.8 for ovine and 8,980.5 eV for bovine (Figure 36). The shift in energy observed alongside increasing oxidation state is due to an electrostatic effect from an increased binding energy of the 1s electron (Waychunas *et al.*, 1983). A shift of ~2-4 eV is typically observed with each increase in oxidation state (Waychunas *et al.*, 1983).

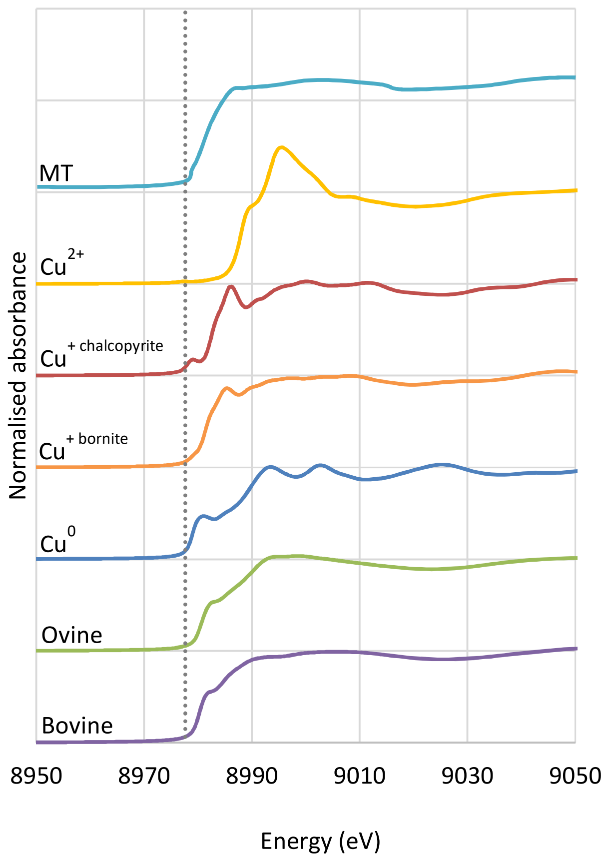

Figure 36: Cu K-edge XANES spectra in energy (μE) for bovine and ovine liver samples versus reference spectra; Cu^0 metallic copper foil, bornite ($Cu^{+}_5Fe^{2+}S_4$), chalcopyrite ($Cu^{+}Fe^{3+}S_2^{2-}$) and copper sulphate ($Cu^{2+}SO_4{\cdot}5H_2O$), alongside XANES spectra data reported by Abrahams *et al.*, for 6Cu:3ZnMT. Dashed line indicates E^0 value for copper K-edge at 8979.3 eV (Abrahams *et al.*, 1986; Thompson *et al.*, 2009).

In addition to oxidation state the character of the ligands which surround the core atom can influence the effective charge on the metal ion; accounting for variation in the edge position for ions in the same oxidation state. Resulting in ions which have the same oxidation state falling within a range, rather than an exact, eV value (Waychunas *et al.*, 1983). The slight difference in E^0 and the spectral shape reported for the ovine and bovine liver samples indicates a slight difference in local co-ordination bonding. The spectral shape reported in these samples is consistent with that of three co-ordination (Sarangi, 2010), and is also consistent with the work of Abrahams *et al.*, (2015) who found that copper metallothionein from pig liver was likely three co-ordinated. The work of D'Angelo *et al.*, (2005) also supports this as they generated remarkably similar XANES spectra from three co-ordinated sulphur ligands and Cu^+. The fitted XANES spectra from the work of Abrahams *et al.*, (2015) shows remarkable similarity to the shape and structure of the sampled livers, although not identical, it does suggest a strong likelihood that the majority of copper is bound to metallothionein in the liver of copper-loaded ruminants (Figure 36).

A reference compound was created for cuprous-thiomolybdate with the intention of fitting to the liver spectra. However, analysis of the XANES spectra in μE was virtually identical for copper sulphate and the created cuprous-thiomolybdate, and only slight differences, consistent with experimental error, existed between the two data sets where they were plotted in R-space (Figure 37).

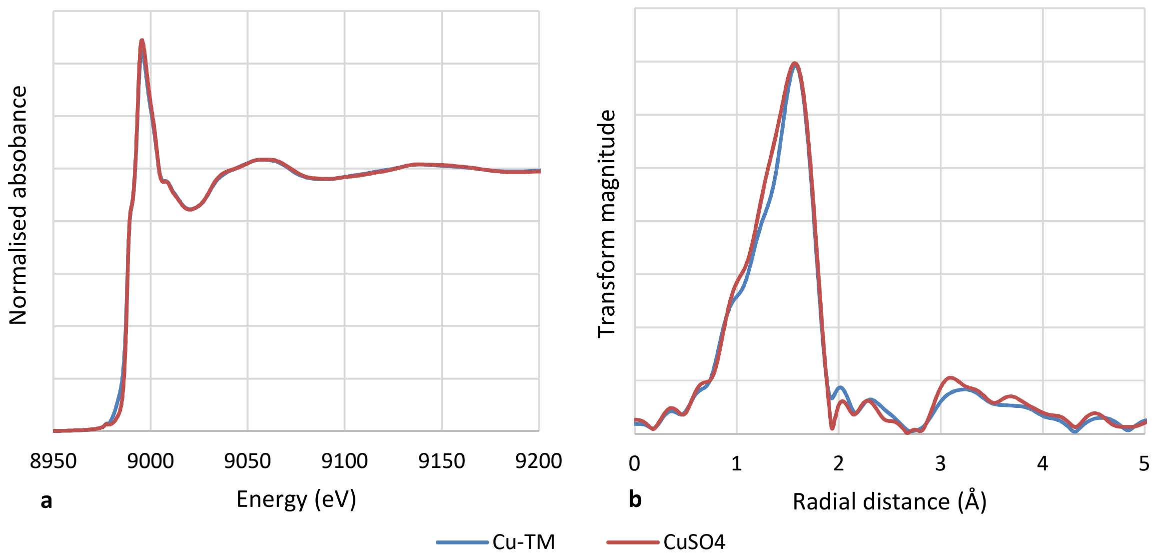

Figure 37: Copper sulphate and cuprous-thiomolybdate plotted in μE (a) and R-space (b). $CuSO_4$ copper sulphate, Cu-TM copper-thiomolybdate.

XAFS peak fitting was performed on the copper sulphate which created a good fit (R= 0.012, S_0^2 0.9, E_0 0.98) in comparison to the standard crystallography data for the compound used (Groom *et al.*, 2016). The same fit was also performed on the cuprous-thiomolybdate data which also confirmed a good fit for the reference data for copper sulphate (R= 0.007, S_0^2 0.8, E_0 1.9) (Figure 38). This confirms that the copper sulphate in the present study is characteristic of the established reference data and strongly suggests that the cuprous-thiomolybdate did not differ to copper sulphate in this experiment.

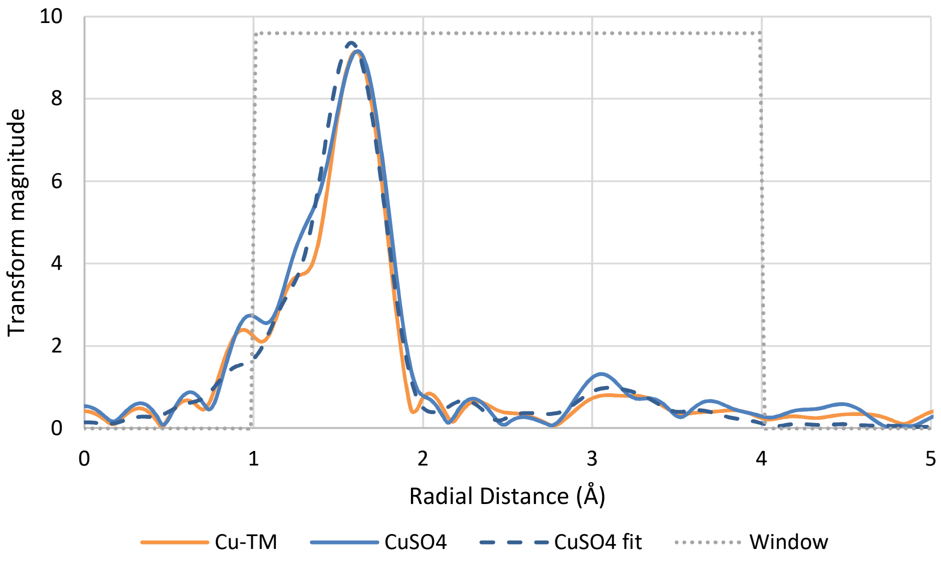

Figure 38: XAFS peak fitting for data sets from copper sulphate, copper-thiomolybdate and reference data for copper sulphate pentahydrate (Groom *et al.*, 2016).

Linear combination fitting (LCF) was used to determine the contribution made by copper sulphate to the copper-thiomolybdate spectra in μE and χ(k). Copper sulphate contributed 90% (±0.5%) of the fit (R= 0.0005) in μE which rose to 99% (±0.4%) contribution in χ(k) (R= 0.023). Supporting the hypothesis that this spectra was mostly comprised of copper sulphate in this experiment and could not be used for fitting to the liver data. This was corroborated though principle component analysis (PCA) which gave copper sulphate as a certain constituent.

The XANES spectra for copper sulphate was notably different to the bovine and ovine spectra. Suggesting in this instance there was no similarity between the liver data and the reference samples (Figure 39). Although, the contribution from copper-thiomolybdate could not be confirmed in this experiment due to the lack of suitable reference sample. The spectra appeared similar to spectra gained by other workers from tetra-thiomolybdate treated Long-Evans Cinnamon (LEC) rat livers; which are commonly used a model for the genetic copper-accumulating condition Wilson's disease in humans (Zhang *et al.*, 2009).

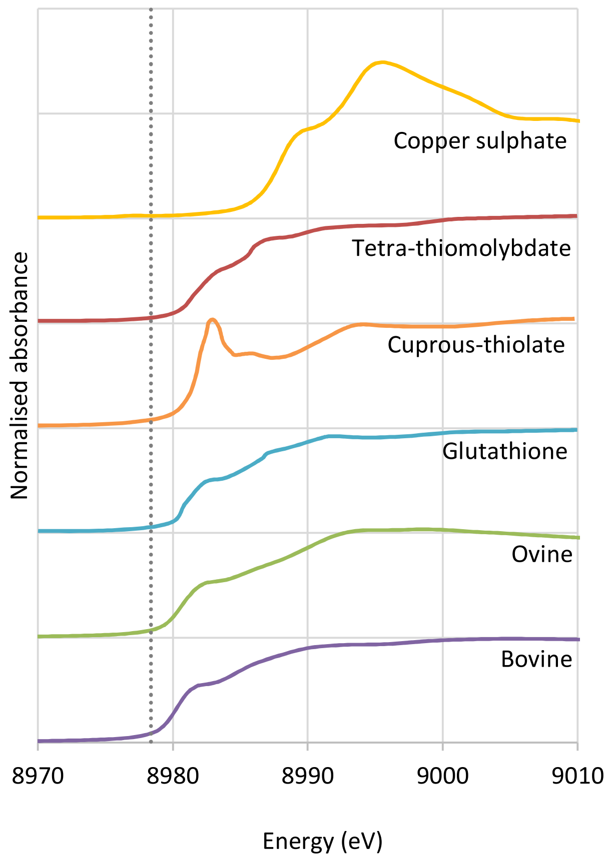

Figure 39: Cu K-edge XANES spectra in energy (μE) for bovine, and ovine liver samples, XANES spectra data reported by Ceko, Aitken and Harris for Cu^{+} Glutathione, Zhang for standard cuprous-thiolate compound $S_2MoS_2CuS_2MoS_2$ from *D. gigas* orange protein, and tetra-thiomolybdate treated LEC rat liver (Zhang *et al.*, 2009), against copper sulphate.

The comparison of the bovine and ovine liver spectra to the tetra-thiomolybdate treated LEC rat spectra remain consistent with the previous conclusions of a three co-ordinated thiolate bond with the copper in its Cu^{+} oxidation state (George *et al.*, 2003; Zhang *et al.*, 2009). However, the similarity in thiolate bonding between metallothionein and tetra-thiomolybdate mean that the contribution of thiomolybdate to hepatic liver accumulation in these species cannot be determined.

6.4.2 XAFS

The intra-species variation in XAFS spectra were close to identical in overall shape and magnitude. Merging of data sets which are close but non-identical can introduce a source of error and dilute peaks. Only data sets which were truly identical after normalisation were merged. However, the overall similarity in data sets indicated that there was little variation between the individual livers of the same species and that the merged, normalised data was representative of the best data set available. Fourier transform magnitude of the sample data plotted in R-space showed some differences between the normalised data for the different species (Figure 40).

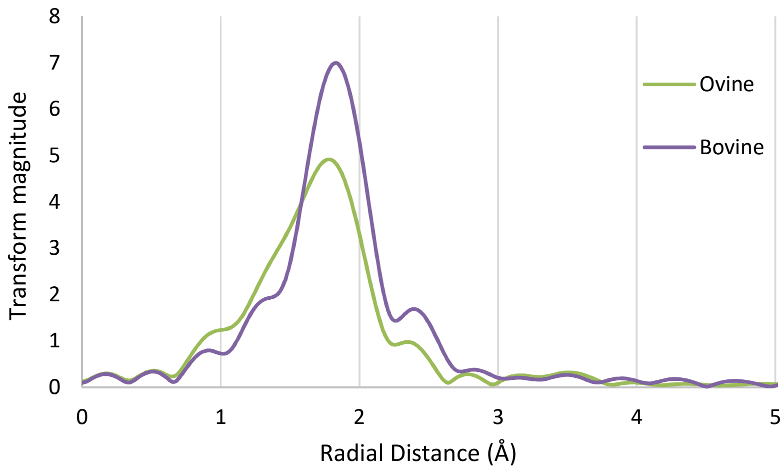

Figure 40: Normalised Fourier transformed data plotted in R-space for bovine and ovine livers with high copper.

The difference in peak magnitude and first peak shape between the species indicates that there is only small difference in the local co-ordination bonding which surrounds the copper atom in the livers of these two species. Analysis of the XAFS data plotted in K-space demonstrated the signal to noise ratio present past 8 Å would negatively impact model fitting results beyond this point. K^2 weighted XAFS data were peak fitted between 1-4 Å for each of the data sets across both bovine and ovine livers (Figure 41 and tabulated in Table 9). Beyond this point the peaks became indistinguishable. Peak fitting was then compared across all data sets for goodness of fit.

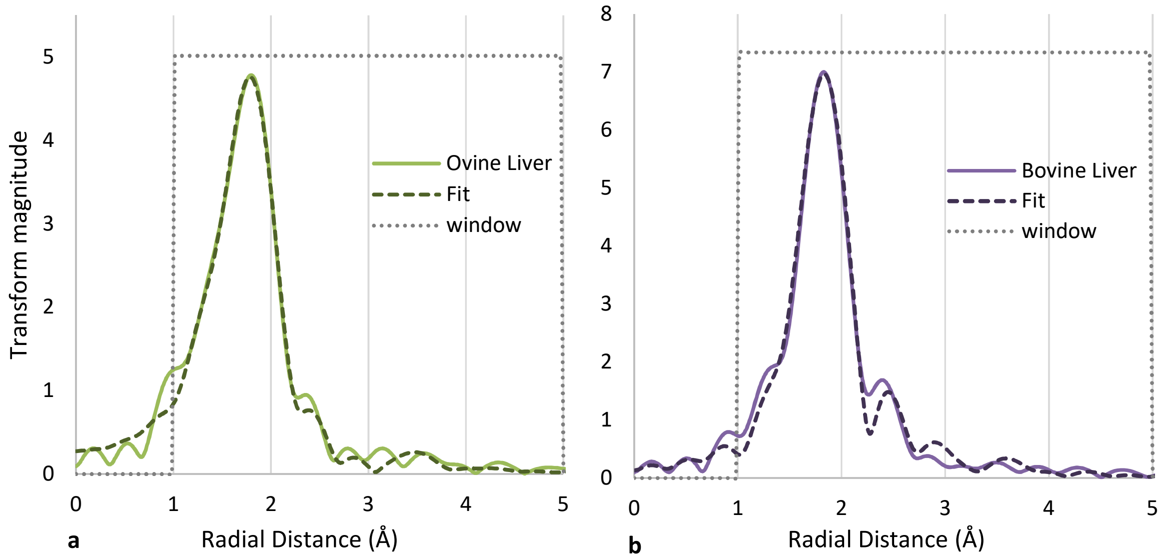

Figure 41: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for ovine (a) and bovine (b) livers with high copper. Solid line denotes sample. Dashed line denotes peak fit. Dotted line denotes window for peak fitting used in model.

The Fourier transform magnitudes are very similar for both species. The peak spectra differs most in magnitude in the first peaks, with key features appearing at similar distances. The spectra were dominated by an initial peak. This first shell in both species fitted with a single scattering sulphur (n=3) pathway. The fitted Cu-S interatomic distance at 2.25 Å in this shell is supportive of three co-ordination (George *et al.*, 2003), possibly a thiolate bond. The first shell fit was enhanced in the sheep liver by the addition of a low magnitude single scattering nitrogen at 1.97 Å. The second shell which created the shoulder at ~2.4 Å was fitted with single scattering carbon atoms (n=4). The third shell showed far less magnitude and fitted with the continued scattering path from the 3rd shell carbons in addition to a further single scattering carbon pathway (n=3-4) at 3.5-3.8 Å. The final peak was also fitted with single scattering carbon (n=6-8). This created good fits across both bovine and ovine liver data (Table 9).

Table 9: n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ²(Å²) for best fit data for bovine and ovine livers. R-factor indicates goodness of fit (<0.02).

Sample	Path	n	S_0^2	R(Å)	Co-ordination shell σ²(Å²)	E_0	Reduced χ²	R-factor
Ovine Liver	Cu-N	1	1.2	1.972	0.016 (±0.007)	3.98	923	0.004
	Cu-S	3	-	2.248	0.014 (±0.002)	-	-	-
	Cu-C	4	-	2.89	0.015 (±0.003)	-	-	-
	Cu-C	3	-	3.837	0.022 (±0.009)	-	-	-
	Cu-C	8	-	4.257	0.029 (±0.006)	-	-	-
Bovine Liver	Cu-S	3	1.2	2.248	0.009 (±0.0004)	5.19	7496	0.01
	Cu-C	4	-	2.884	0.007 (±0.003)	-	-	-
	Cu-C	4	-	3.483	0.016 (±0.008)	-	-	-
	Cu-C	6	-	4.257	0.02 (±0.008)	-	-	-

Alternatively, a single scattering molybdenum could be fitted at 2.65 Å in place of the carbon in both the bovine and ovine livers; creating a good superficial peak fit in both species. The fit for the bovine livers (Figure 42 and tabulated in Table 10) needed to be constrained for S_0^2 and E_0 indicating that this fit was not ideal. However, in the ovine livers (Figure 42, Table 10) the fit was statistically meaningful; generating an equally likely fit. Suggesting that thiomolybdate may be present in the ovine liver samples, alongside metallothionein or possibly as part of complex containing both ligands.

Table 10: n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ²(Å²) for best fit data ovine liver with molybdenum fitted in the second shell. R-factor indicates goodness of fit (<0.02). Asterisk indicates constrained factor.

Sample	Path	n	S_0^2	R(Å)	Co-ordination shell σ²(Å²)	E_0	Reduced χ²	R-factor
Ovine Liver	Cu-N	1	0.96	1.972	0.009 (±0.004)	3.63	817	0.003
	Cu-S	3	-	2.248	0.01 (±0.002)	-	-	-
	Cu-Mo	1	-	2.653	0.016 (±0.005)	-	-	-
	Cu-C	2	-	2.901	0.012 (±0.006)	-	-	-
	Cu-C	3	-	4.153	0.019 (±0.007)	-	-	-
Bovine Liver	Cu-S	3	1.2*	2.248	0.008 (±0.0003)	10*	3888	0.006
	Cu-Mo	1	-	2.653	0.01 (±0.001)	-	-	-
	Cu-C	2	-	2.901	0.016 (±0.006)	-	-	-
	Cu-C	1	-	4.153	0.038 (±0.062)	-	-	-

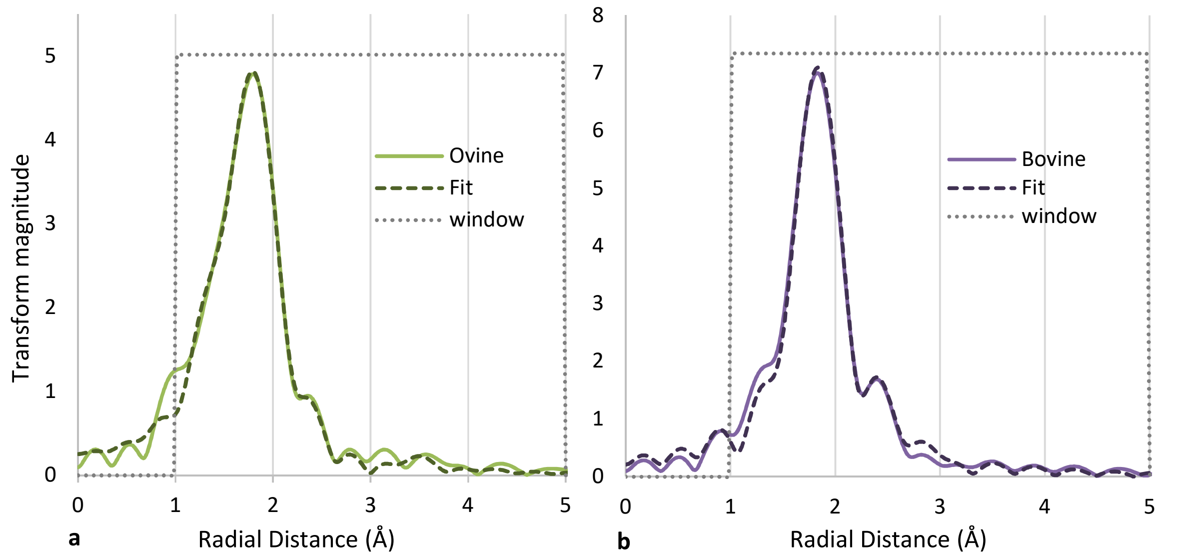

Figure 42: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for tabulated fits of ovine liver (a) and bovine liver (b). Solid line denotes sample. Dashed line denotes peak fit. Dotted line denotes window for peak fitting used in model.

6.5 Discussion

Due to the nature of XAS analysis and the independent nature of spectral interpretation and modelling the findings are best discussed individually.

The XANES spectra and E^0 values in the current study are consistent with the presence of copper in its monovalent (Cu^+) oxidation state in the hepatic compound of both cattle and sheep. Monovalent copper is the primary substrate for intercellular transport, despite its divalent (Cu^{2+}) form being more soluble (Prohaska, 2008). Within the intestines Cu^{2+} can be reduced into Cu^+ through catalysis using reductases in the plasma membrane (Kaneko *et al.*, 1997; Tennant *et al.*, 2002; Thiele, 2003) and in most biological compounds Cu^+ is the prevalent form (Kaneko *et al.*, 1997; Failla, 1999; Suzuki *et al.*, 2002; Leary *et al.*, 2009; Spears, 2013; Verwilst *et al.*, 2015).

The XANES spectra alongside the XAFS peaks support the hypothesis for three co-ordinated sulphur in both species livers. This is also consistent with the Hard-Soft Acid-Base (HSAB) theory. Cu^+ is a much softer Lewis acid (an ionic compound which can accept an electron pair) than Cu^{2+}, which is considered borderline between hard and soft, Cu^+ has a lower charge with a larger ionic radius (Helsel and Franz, 2015). The soft-soft interaction of Cu^{+} and sulphur will occur more rapidly and form a stronger bond than Cu^{2+} with sulphur (Rizvi *et al.*, 2015). This is also seen in biological thiol complexes as Cu^{2+} complexes with glutathione and cysteine are much less stable than Cu^{+} complexes relating to the soft Lewis acid character of Cu^{+} (Le Gall and Van den Berg, 1993). Glutathione is known to reduce Cu^{2+} into Cu^{+} during complex formation (Leal and Van Den Berg, 1998), this suggests that in Cu^{+}-thiol complexes, Cu^{2+} is first reduced to Cu^{+} in the presence of the thiol, and then complexes, as a result of the specific complexation of Cu^{+} (Leal and Van Den Berg, 1998).

The XANES spectra from both species livers share distinct similarity with the previously gathered glutathione spectra (Ceko *et al.*, 2014). Although, this is not an identical spectra it may be indicative of a small proportion of copper binding to glutathione in the copper-loaded ruminant liver. Copper is able to bind to glutathione, a small cysteine containing tripeptide, at a much lower affinity than metallothionein. Within the cell, glutathione appears to shuttle imported copper onto its specific chaperones for production of cellular cuproenzymes and cuproproteins (Tapiero *et al.*, 2003; Nevitt *et al.*, 2012). Glutathione is present in almost every cell at concentrations 1-10 mM (Forman *et al.*, 2009), and it may act as a low-molecular ligand to stabilise Cu^{+} in the intracellular environment (Palumaa, 2013). Binding to glutathione could represent a potential buffering role preventing cellular damage in the absence of sufficient metallothionein transcription (Nevitt *et al.*, 2012; Verwilst *et al.*, 2015).

The analysis of the XAFS and XANES spectra for the bovine and ovine liver samples is consistent with previous work suggesting that copper in mammalian livers is predominantly in the form of metallothionein. XAFS analysis of rat liver metallothionein has previously provided evidence of three co-ordination in the first shell by single scattering sulphur atoms at copper-sulphur distances of 2.25 ± 0.03 Å with amplitudes and distances consistent with trigonal co-ordination (Abrahams *et al.*, 1986; George *et al.*, 1986), which creates a match with the sulphur fitted in this study at 2.248 Å in combination with a low magnitude single scattering nitrogen ligand at 1.97 Å in the ovine samples. Nitrogen is also present in cysteine residues and is consistent with other copper ligands in biological complexes (Miroslaw *et al.*, 2019). The similarity between the current XANES spectra and metallothionein from previous studies (Abrahams *et al.*, 1986) is also a close, but imperfect fit; potentially a combination of metallothionein and glutathione. Both of which produce similar spectra due to the thiol co-ordination in the first shell surrounding the copper atom. Although present in all cells, metallothionein is particularly found in the liver (discussed in Section 6.1.1). Figure 43 demonstrates the similarity in copper co-ordination bonding for cysteine residues from both glutathione and metallothionein.

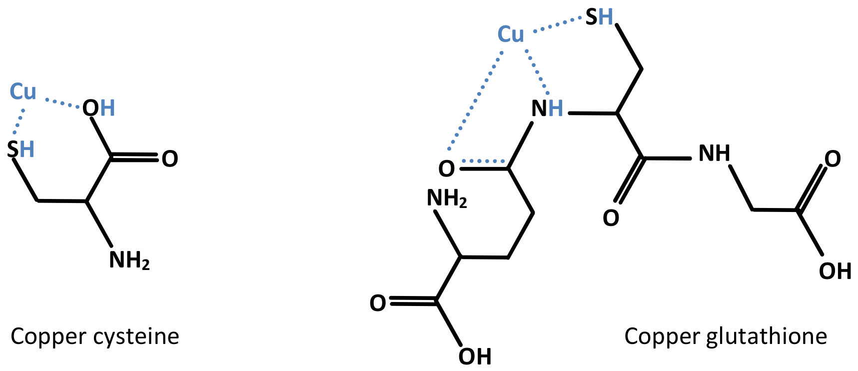

Figure 43: Copper binding sites to cysteine residues from glutathione and metallothionein. Metallothionein is a small protein ~6 kDa in size which contains 61-62 amino acid residues, of which 20 are cysteines. The common motif CysXCys tripeptide sequence, where X represents a non-cysteine amino acid, provides the copper binding site (Bertini *et al.*, 2007). Blue indicates copper binding pathway and dashed line denotes changes to the molecule with copper binding.

Cellular studies in other mammals have demonstrated that copper chaperones and copper-binding proteins handle cytosolic copper using cysteine-thiols. These are ideal ligands for intracellular Cu^+ as they are able to create a stable Cu^+-thiol complex which protects the cell from oxidative damage while allowing transfer to copper acceptors for functional use (Hatori *et al.*, 2017). The binding sites on the copper chaperones are often of a trigonal planar geometry and have co-ordination numbers of 2 or 3 consistent with the findings in the present work (Hatori *et al.*, 2017).

The lysosomal store of metallothionein bound copper is thought to act as a buffer for shifts in cellular copper concentration (Kaneko *et al.*, 1997; Dameron and Harrison, 1998; Failla, 1999; Thiele, 2003). The expression of metallothionein, regulated by MTF1, is rapidly induced in response to elevated copper in monogastric species (Suzuki *et al.*, 2002). But, the limited metallothionein response of ruminants prevents this rapid binding of copper in elevated copper conditions and inhibits the mobilisation of stored copper when copper is lacking, especially in sheep (Saylor *et al.*, 1980; Bremner and Beattie, 1995; Nevitt *et al.*, 2012), discussed in Section 6.1.4. Although, the data in this work supports metallothionein as the most likely compound in both bovine and ovine livers of high copper status the presence of a single scattering nitrogen pathway which was absent in the cattle livers could implicate glutathione as a buffer for excess copper beyond the metallothionein carrying capacity in sheep; where their metallothionein synthesis and biliary excretion is unable to keep up with rising copper (Saylor *et al.*, 1980; López-Alonso *et al.*, 2005a). The subsequent accumulation of copper would cause and cellular damage if it is not chaperoned; giving glutathione a potentially vital role in this species.

The data demonstrates that there is a notable difference between the bovine and ovine liver copper compounds. XAS represents only the average structure surrounding the core atom, as such, differences between the two species are harder to distinguish where they share a contributing major compound. Both livers carry a large degree of similarity, which suggests their major copper-containing compound is synonymous for both species. Although, biological differences in metallothionein transcription and their ability to cope with an increasing cellular concentration of copper could be responsible for the observed differences (Corbett *et al.*, 1978; López-Alonso *et al.*, 2005a, 2005b). Previous work has established that in sheep the abundance of metallothionein, as a result of a reduction of metallothionein mRNA, declines markedly after birth. Contributing to the observed inhibited response to rising copper. This has led to the observation of very little copper associated with metallothionein in copper-loaded sheep in contrast to rats or pigs and found that only a small proportion for cytosolic copper was bound to metallothionein (Mehra and Bremner, 1984; Paynter *et al.*, 1990).

In many high copper status livers, copper accumulation may not simply be a direct effect of oversupply. If systemic thiomolybdate absorption has taken place, it has not yet been established if the copper accumulating in the liver is bound to a thiomolybdate complex, which may have the potential to interrupt its function and reduce its bioavailability. The livers used in this work were abattoir recovery samples and samples submitted for diagnostics and therefore had not been previously fed controlled diets. ICP-MS analysis confirmed the livers had molybdenum concentrations towards the upper end of normal range (5-52 µmol/kg DM bovine, 11-45 µmol/kg DM ovine, NUVetNA reference data, 2019). Subsequently, it is not possible to determine if these samples had systemic thiomolybdate present. The XANES spectra obtained showed some similarity to previous data from LEC rat livers exposed to tetra-thiomolybdate after hepatic copper accumulation, but not enough to conclude its presence (Zhang *et al.*, 2009). This previous work was deemed an example of hepatic copper chelation by thiomolybdate (Zhang *et al.*, 2009), but the current data lacks the peak towards the top of the rising edge (~8986 eV) to be analogous and the appropriateness of LEC rat data in this case can be debated (Section 9.3.4). The oxidation state as Cu^+ observed in the present work would be conducive to thiomolybdate binding and the XAFS peak fitting of three co-ordinated sulphur found in this work would also be consistent with thiomolybdate (Zhang *et al.*, 2009), which is known to bind to copper in three co-ordination through one thiol group and two sulphur atoms which bridge to molybdenum (George *et al.*, 2003; Alvarez *et al.*, 2010; Helz and Erickson, 2011). The molybdenum bond in at 2.7 Å from the copper atom in the previous work was consistent with the molybdenum fitted in this trial. However, in this case it is unlikely that the bovine hepatic storage compound had any notable amount of copper-thiomolybdate since the second shell did not create a good fit with the molybdenum atom without mathematical constraint. However, the ovine liver did create a good fit for copper-thiomolybdate and despite the normal concentrations of molybdenum in the liver, the differences in XAFS spectra and inhibited metallothionein response in the species mean that the storage of some copper as copper-thiomolybdate in the ovine liver cannot be ruled out.

6.6 Conclusion

The oxidation state of copper in the liver of both cattle and sheep is likely to be Cu^{+}. Livers containing high concentrations of copper from both species showed remarkable similarity in their XAFS and XANES spectra to those of copper-metallothionein from monogastric species. This supports the notion that metallothionein is the main storage compound for copper in the liver and is consistent with the observed oxidation state. Although, the data did not generate a perfect fit to this spectra. There was also a high degree of similarity to glutathione and copper from a thiomolybdate treated LEC rat liver. There was only a small difference between the local co-ordination bonding for the two species and both livers appeared to be three co-ordinated by thiols, consistent with each of the potential contributing compounds. This suggests, that the copper in the liver may be a mixture of these compounds; although the similarity in the thiol bonding of these compounds makes differentiation more difficult. In sheep, there was a likelihood of a greater role for glutathione and it was also possible that copper was present bound to thiomolybdate in this species.

7.0 XAS modelling of copper compounds in digesta

7.1 Introduction

Interesting similarities exist between the localised environmental conditions created in euxinic water and the rumen. Euxinic water occurs where parts of the sea become isolated from the main body of water. The water volume is maintained through an inflow that does not exceed evaporation (Meyer and Kump, 2008). The isolation results in varying degrees of stagnation and stratification within the body of water. It is debated how much euxinic water has existed in the past, but it is currently limited to localised areas in the Norwegian fjords and the Black Sea (Meyer and Kump, 2008).

Euxinic water is characterised by being anoxic with an elevated, stable concentration of sulphur (~7 mM), predominantly as hydrogen sulphide within the water column (Canfield, 2011). Often located beneath a layer of oxygen containing (oxic) surface water. The absence of oxygen, alongside the presence of; sulphate ions, organic matter, a population of sulphur-reducing bacteria, and molybdenum; provided through the weathering of rock washing into the ocean, are all effective criteria to establish the presence of euxinic water (Algeo and Maynard, 2004). A lack of copper solubility has also been observed under euxinic conditions.

The rumen (discussed in Section 1.5), like euxinic water is a reducing environment, they both have a liquid and sedimentary region and possess sulphur-reducing bacteria, as well as an elevated sulphur content and anoxic conditions.

Interestingly, thiomolybdate naturally occurs in euxinic water. Two remarkably stable thiomolybdate-Cu^{+} dissolved complexes have been identified at 23°C ($Cu_2(HS)_2MoS_4$ and $Cu_2S_2MoS_4$) (Helz and Erickson, 2011), although more species may exist. Molybdenum present in oxic water as molybdate ions are considered unreactive, but in euxinic environments, molybdenum precipitates into thiomolybdate (Arnold *et al.*, 2004). Much of the research discussed in Section 6.1.5 has been drawn from studies in euxinic water. The research conducted on the behaviour and chemistry of thiomolybdate and modelling work using copper-thiomolybdate reinforces the concept that the oxidation state is integral to the chemistry, complexing and stability in euxinic water (Clarke and Helz, 2000; Erickson and Helz, 2000; Helz and Erickson, 2011). Since the rumen shares many of these similarities this research helps to explain the characteristics which have been observed using rumen fluid and *in vitro* models (Quagraine, 2002; Essilfie-Dughan, 2007). The oxidation state of copper is of paramount importance to its absorption, transport and biological function (Clarkson *et al.*, 2019). Despite this, the oxidation state of copper and its antagonists have not been investigated in *in vivo* samples.

Euxinic water may not only be useful in explaining the mode of action for thiomolybdates with copper, there may also be useful insight that can be used to assist in furthering understanding of the iron, sulphur, copper antagonism.

Iron is also abundant in euxinic water and several compounds exist in geology which contain copper, sulphur and iron. Of these compounds the two most abundant copper-iron sulphides in nature are chalcopyrite ($CuFeS_2$) and bornite (Cu_5FeS_4). These compounds are able to form in a wide range of conditions and will precipitate at moderate-temperatures where mineral-rich hydrothermal fluid enters the water system (Zhao *et al.*, 2014). The similarity between their forming environments and composition of the compounds makes them interesting candidates to study in relation to the unknown iron-copper-sulphur compound in the rumen.

7.2 Aims & Objectives

The work in this chapter aims to determine the oxidation state of copper and iron in the digesta located in rumen and jejunum of sheep fed a high copper, sulphur and iron diet. Secondly, the work aims to provide information on the local co-ordination bonding of copper and iron in these digestive regions.

7.3 Materials & Methods

7.3.1 Animal experimental design

Three 9-10 month old Charollais x lambs (BW ~35 kg) were housed on inedible bedding (HempCore, Aubiose, Buckingham, UK) for a period of 5 days and fed a dried-grass only diet

(0.8 kg/day, Graze-on, Northern Crop Driers, York, UK). After this acclimation period, the sheep were additionally fed a daily treatment containing high iron, copper and sulphur from reagent grade sources (Table 12) mixed into 20 g of molasses (NAF, Monmouth, UK); which was accounted for in the basal diet calculation (Table 11), for a period of 48 h prior to slaughter. This short time frame was used in order to 'load' the digestive tract with copper and its antagonists at a concentration sufficient to meet the detection limits of the beamline whilst minimising any risk of acute toxicity arising from the absorption of copper.

Table 11: Mineral content of the basal diet for controlled elements as analysed by ICP-MS ± standard deviation.

	Iron mg/kg DM	**Molybdenum mg/kg DM**	**Copper mg/kg DM**	**Sulphur g/kg DM**	**% of total diet**
Dried chopped grass	230±13.9	1.7±0.05	6.1±0.00	3.2±0.01	90.5
Molasses	205.3±115.7	2±0.95	22.5±13.6	12.9±7.8	9.5
Concentration in complete basal diet	**227.7**	**1.7**	**7.7**	**4.1**	**100**

Table 12: Inclusion rates and details of elemental source for the experimental diet.

	Iron (mg/kg DM)	**Copper (mg/kg DM)**	**Sulphur (g/kg DM)**
Basal diet	229.5	6.5	3.4
Treatment (mg/day)	200	30	480
Total inclusion	**500**	**40**	**4**
Compound & oxidation state	$Fe^{3+}Cl3 \cdot 6H2O$	$Cu^{2+}Cl_2 \cdot 2H_2O$	Na_2SO_4
Purity (%)	>99	≥99.0	>99
Manufacturer	Fisher Scientific, Loughborough, UK	VWR International Ltd, Lutterworth, UK	VWR International Ltd, Lutterworth, UK

At slaughter, the digestive tract was carefully removed without puncturing and was sectioned off using two cable ties to secure the junction and cut in between them to section the tract. The following regions were obtained; rumen, reticulum, omasum, abomasum, duodenum and jejunum. Digestive sections were snap-frozen at the time of culling using an ethanol (>99%, VWR International Ltd, Lutterworth, UK) and solid carbon dioxide (Air Liquide, Stoke-on-Trent, UK) bath (-80°C). The frozen sections were maintained at -20°C once completely frozen and cut into smaller sections whilst still frozen using a band saw. The frozen digesta sections from the rumen and jejunum were then cut using a scalpel into blocks ~10 g WW. The samples were then subject to wet-acid digestion and ICP-MS to determine elemental composition (using methods described in Sections 5.3.3.2 and 3.3.2.1) and freeze-dried, ground and prepared into sample holders using the method described in Section 6.3.1 with the removal and discarding of any tissue matter after freeze-drying using forceps.

7.3.2 Standard preparation

Standards for the copper K-edge were the same as those used in Section 6.3.2. Standards for the iron K-edge were run in transmission mode and comprised chalcopyrite and bornite, prepared as previously described (in Section 6.3.2), alongside reagent grade iron sulphate ($Fe_2^{3+}(SO_4)_3{\cdot}9H_2O$, VWR, Leicestershire, UK. >99%) prepared into pellets using the same process. Iron metallic foil (I20, Diamond Light Source, Oxfordshire, UK) was also analysed in transmission mode in line with the incident beam alongside the standard samples.

7.3.3 XAS Analysis

XAS data for the samples was collected in fluorescence mode at beamline I20-Scanning at the Diamond Light Source (Oxfordshire, UK) using the method previously described in Section 6.3.3 for copper K-edge. Iron K-edge data was collected at 7,112 eV.

7.3.4 Data analysis

All XAS scans, transmission and fluorescence, were analysed using the process described in Section 6.3.4 via the Demeter software package (Ravel and Newville, 2005).

7.4 Results

7.4.1 XANES of the copper edge

The E^0 of the rumen digesta indicates that the average oxidation state of copper in both the rumen and jejunum samples is likely to be between Cu^0 and Cu^+. As previously discussed (Section 6.4.1), Cu^+ edge positions range from 8,983-8,984 eV increasing to >8,985 eV for Cu^{2+} (Ceko *et al.*, 2014). The E^0 for both rumen and jejunum samples lie very close to the E^0 of metallic copper 8,979.3 eV (Thompson *et al.*, 2009), at 8,980 and 8,981 eV respectively. Which is likely to represent some copper in its Cu^+ oxidation state alongside the presence of a notable proportion of Cu^0; elemental or metallic copper.

The XANES spectra are identical for the pre-edge and edge regions between the rumen and jejunum samples when plotted in energy (μE). The only difference is a slightly lower magnitude in the jejunum samples at the edge maxima alongside a slight shoulder at the crest at 8,997 eV which is absent in the rumen samples (Figure 44). However, the spectra differ greatly in the post edge region (8,995-9,374 eV) especially when the data is plotted in R and K space. The spectra generated for both digesta samples is more consistent with copper in a three or four co-ordinated geometry, most likely in a tetrahedron (Sarangi, 2010). shows the XANES spectra for digesta data versus standard and reference samples.

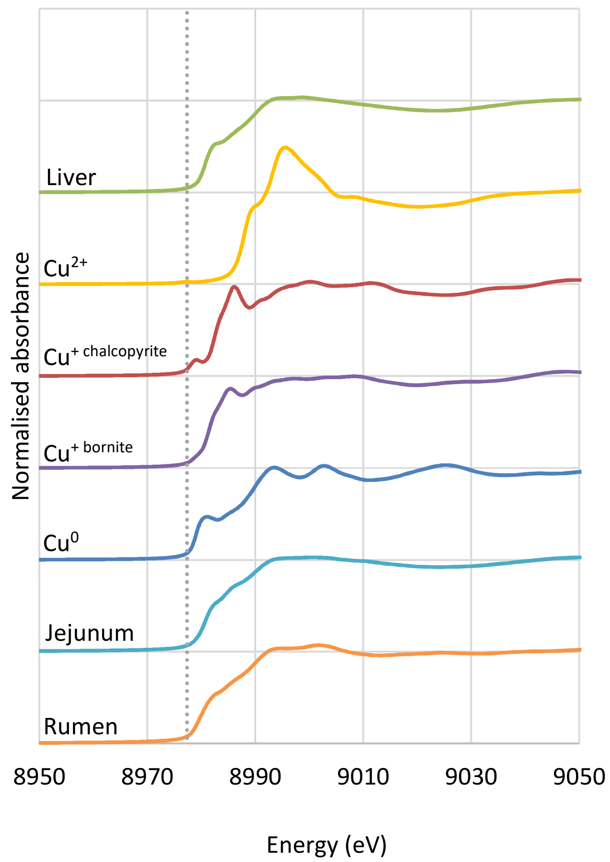

Figure 44: Cu K-edge XANES spectra in energy (μE) for ovine digestive samples taken from the rumen and jejunum versus reference spectra; Cu^0 metallic copper foil, bornite ($Cu^{+}_5Fe^{2+}S_4$), chalcopyrite ($Cu^{+}Fe^{3+}S_2^{2-}$), copper sulphate ($Cu^{2+}SO_4{\cdot}5H_2O$), and the ovine liver data reported in Section 6.4.

By comparing the shape of the spectra, the digestive samples bear a greater similarity to the previously examined liver sample than to copper sulphate and chalcopyrite. Although, there is a small degree of similarity of spectral shape to bornite and metallic copper. The features of metallic copper at 8,981 and 9,026 eV are absent and those at 8,994 and 9,004 eV are largely diminished in the sample spectra. The absence of the edge feature at 8,986 eV present on bornite and at 8,983 eV on the liver sample suggest that the local co-ordination bonding may be similar but not identical to these compounds. From looking at the spectra it is evident that the copper has undergone complete speciation from its initial copper compound; divalent (Cu^{2+}) copper chloride (Figure 45), as evidenced by the energy shift away from Cu^{2+} and changes in the magnitude and spectra generated.

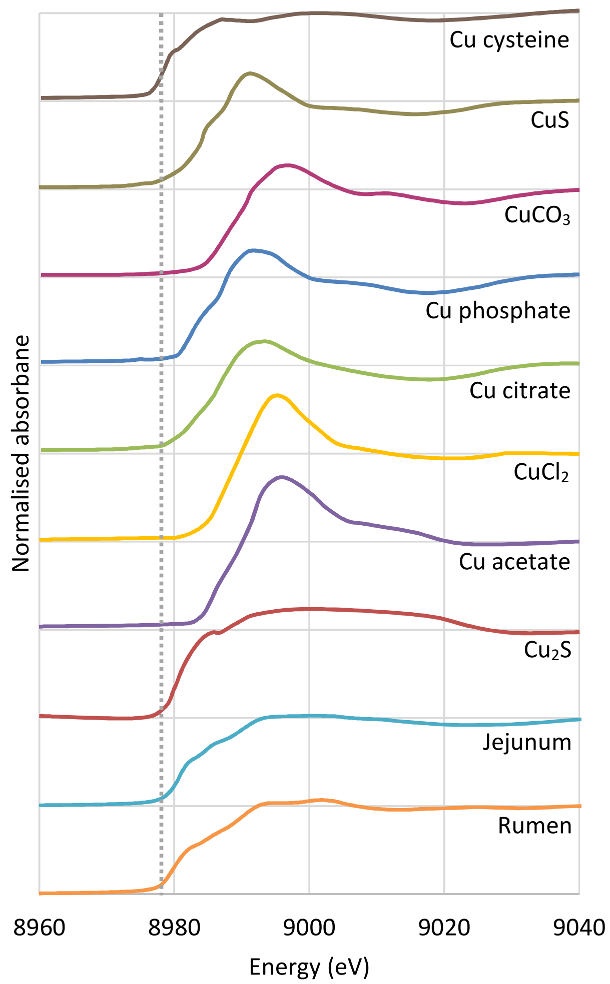

Figure 45: Cu K-edge XANES spectra in µE for ovine rumen and jejunum samples alongside standards; Cu^{+} sulphide (Cu_2S), Cu^{2+} acetate ($Cu(CH_3COO)_2$) from Dimkpa *et al.*, (2012), Cu^{2+} chloride ($CuCl_2$) from Klaiphet *et al.*, (2018), Cu^{2+} citrate ($Cu_3(C_6H_5O_7)_2$), Cu^{2+} phosphate ($Cu_3(PO_4)_2$), Cu cysteine, Cu^{2+} sulphide (CuS) from Chen *et al.*, (2011), Cu^{2+} carbonate ($CuCO_3$) from Sekine *et al.*, (2017).

In comparison to spectra gathered from a wider range of copper containing compounds there is most similarity to copper cysteine. Although the spectral shape is not identical for copper cysteine it does share some resemblance, which may also account for the similarity to the liver samples. A Cu^{+} oxidation state and potential co-ordination to sulphur is supported in comparison to the copper sulphides; as the samples share some approximation to Cu^{+} sulphide and vary greatly from Cu^{2+} sulphide.

7.4.2 XANES of the iron edge

The E^0 of metallic iron lies at 7,112 eV with edges greater than 7,118 indicating Fe^{2+} and those in excess of 7,125 eV likely to represent Fe^{3+} (Waychunas *et al.*, 1983; Monarumit *et al.*, 2016). The E^0 obtained from the iron K-edge in the rumen and jejunum digesta indicates that the average oxidation state of iron is likely to be a mixture of Fe^{2+} and Fe^{3+} as edges for these samples were at 7,124.7 and 7,125.2 eV respectively. A comparison of the spectra alongside the standards confirmed that the spectra fell between the standards for Fe^{2+} and Fe^{3+} demonstrating spectral shape in line with a combination of iron from both oxidation states (Sarangi, 2010). Close comparison of the E^0 and XANES spectra indicates that the rumen samples contain a higher proportion of iron as Fe^{2+} than those in the jejunum. The spectra for the rumen and jejunum samples are very similar when plotted in energy (μE). The E^0 values are very close and the spectral shapes share the same edge features at 7,115 eV. The two spectra are differentiated by slight difference in peak magnitude at ~7,117 eV and a lower magnitude at the edge maxima in the rumen sample consistent with a higher proportion of Fe^{2+} (Figure 46).

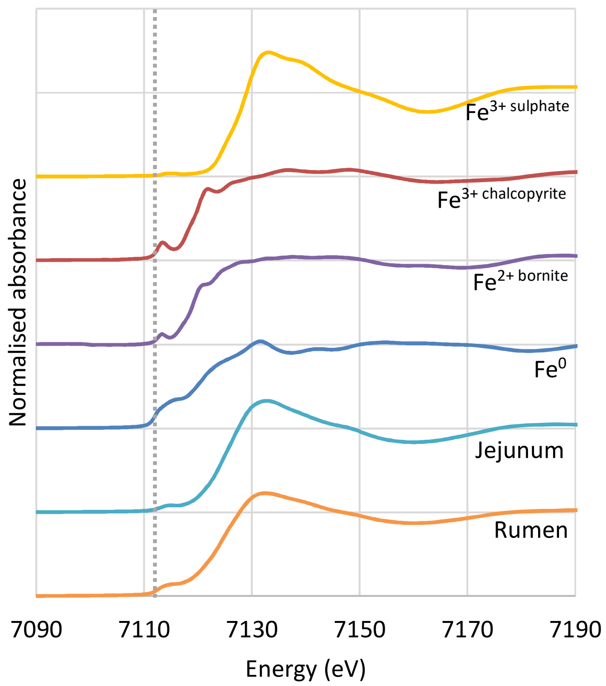

Figure 46: Fe K-edge XANES spectra in µE for ovine rumen and jejunum samples alongside standards; metallic iron Fe^0, bornite[b] ($Cu^{+}_{5}Fe^{2+}S_4$), chalcopyrite[c] ($Cu^{+}Fe^{3+}S_2^{2-}$) and iron sulphate[s] ($Fe_2^{3+}(SO_4)_3{\cdot}9H_2O$). Dashed line indicates E^0 value for iron K-edge at 7112.2 eV (Thompson *et al.*, 2009).

The spectra from the samples only show a single pre-edge feature at ~7,114 eV. A pre-edge in this location is most commonly observed for non-centrosymmetric geometry such as tetrahedral co-ordination. Although, it could possibly represent 5-co-ordination in a trigonal bipyramid (Wilke *et al.*, 2001). Similar to the copper, the iron in the digestive samples differs greatly in XANES spectra and E^0 value from the fed compound; trivalent (Fe^{3+}) iron chloride. Interestingly, the iron appears to resemble the XANES spectra from iron sulphate most closely. Both samples and iron sulphate appear similar in binding energy and spectral shape. Although, the sulphate has a peak of higher magnitude at the edge maxima and the pre-edge features of the samples at ~7,115 eV are more pronounced. The spectra share very little commonality with those of chalcopyrite and bornite. In comparison to a range of compounds from the literature there is very little likelihood that the iron compounds in the digestive samples are represented (Figure 47).

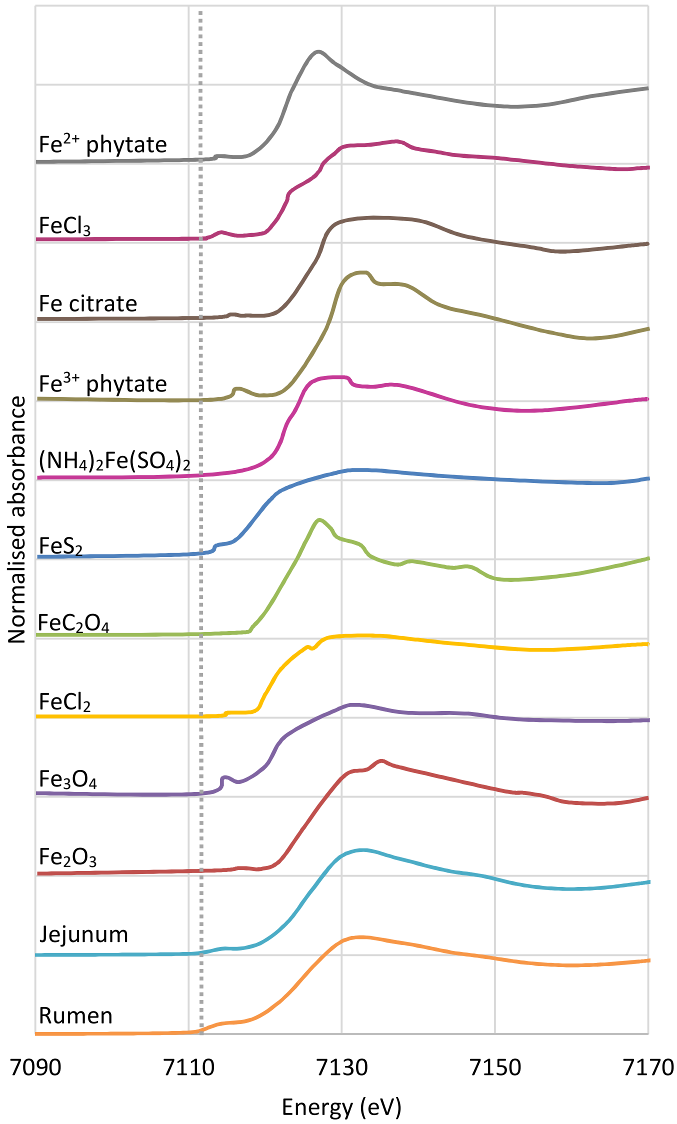

Figure 47: Iron K-edge XANES spectra in energy (μE) for rumen and jejunum samples alongside XANES spectra data reported by Fittschen *et al.*, (2008) for Fe^{3+} oxide (Fe_2O_3), $Fe^{2/3+}$ oxide (Fe_3O_4), Fe^{2+} chloride ($FeCl_2$), Fe^{2+} oxalate (FeC_2O_4), Fe^{2+} sulphide (FeS_2), Ammonium Fe^{2+} sulphate ($(NH_4)_2Fe(SO_4)_2$), Fe^{3+} phytate, Fe^{2+} phytate, Fe^{3+} citrate ($C_6H_5FeO_7$) from Singh *et al.*, (2013) and Fe^{3+} chloride ($FeCl_3$) from He *et al.*, (2012).

Despite this, there is some resemblance in XANES spectra to Fe^{2+} sulphide. Which could suggest a mixture of iron sulphate and sulphide. Although, the spectra differ sufficiently that this cannot be a match, it is consistent with the measured oxidation states of the digestive samples and both the pre-edge features and edge maxima occur at similar energies.

7.4.3 XAFS

7.4.3.1 XAFS of the copper K-edge

The XANES spectra are very similar across both sample types when plotted in energy (μE), however, the spectra vary greatly when plotted in R and K space. This suggests that they may share an oxidation state but the contributing atoms in their local co-ordination and further shells are different. The intra-sample variation in XAFS spectra shared a great deal of similarity but had a degree of overall difference in shape and magnitude. This is likely due to the heterogeneous nature of digesta. Only data sets which were truly identical after normalisation were merged. Fourier transform magnitude of the sample data plotted in R-space showed notable differences between the data for the copper K-edge at different regions in the same digestive tract (Figure 48).

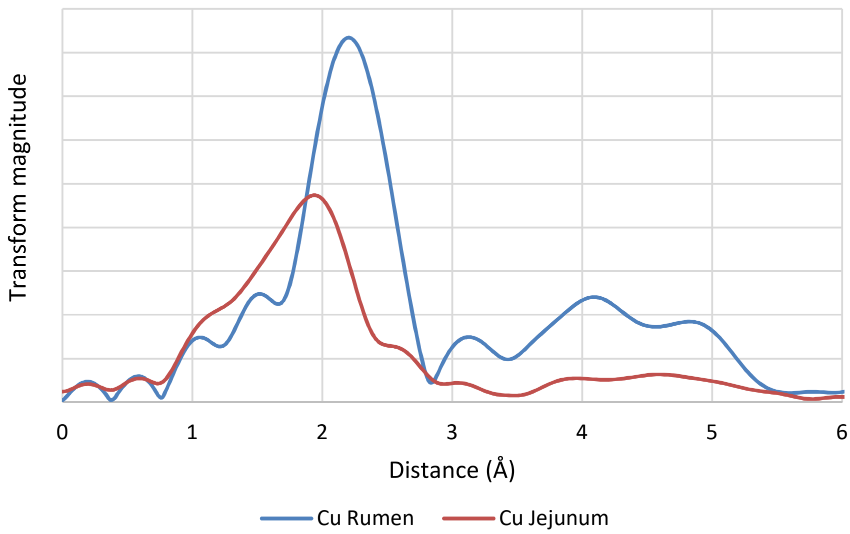

Figure 48: Normalised Fourier transformed data plotted in R-space for copper K-edge data from ovine rumen and jejunum digestive samples.

Analysis of the XAFS data plotted in K-space demonstrated the signal to noise ratio present past 8 Å would negatively impact model fitting results beyond this point. K^2 weighted XAFS data were peak fitted between 1-4.5 Å for the data. Beyond this point the peaks became less distinguishable. Figure 49 shows the normalised and fitted paths. All fitted paths and distances are tabulated in Table 13.

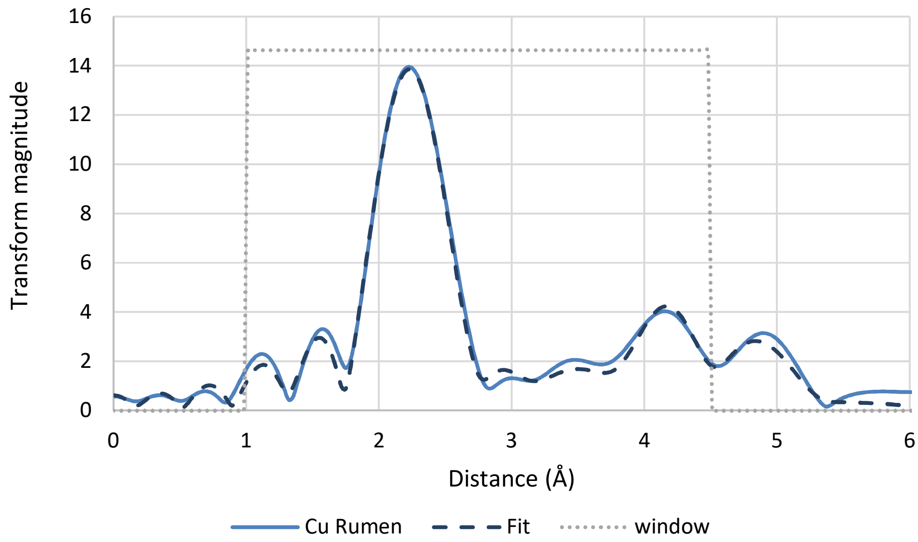

Figure 49: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for copper K-edge rumen digesta. Solid line denotes sample. Dashed line denotes peak fit. Dotted line denotes window for peak fitting used in model.

The initial distance for the first shell was consistent with sulphur and fitted well with tetrahedral co-ordination, consistent with the XANES spectral observations. All other further paths fitted well with additional copper atoms as single scattering or as a double forward scattering pathway at 5 Å.

Table 13: n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ^2(Å^2) for best fit data for rumen copper K-edge. R-factor indicates goodness of fit (<0.02).

				Co-ordination shell				
Sample	**Path**	**n**	S_0^2	**R(Å)**	**σ^2(Å^2)**	E_0	**Reduced χ^2**	**R-factor**
Rumen	Cu-S	4	0.9	2.096	0.184 (±0.023)	3.64	2735	0.019
	Cu-Cu	6	-	2.553	0.008 (±0.0005)	-	-	-
	Cu-Cu	4	-	3.61	0.013 (±0.004)	-	-	-
	Cu-Cu	6	-	4.421	0.006 (±0.002)	-	-	-
	Cu-Cu-Cu-Cu	8	-	5.105	0.012 (±0.025)	-	-	-

The number of fits which were successfully fitted to Cu-Cu were unexpectedly high and showed some similarity to data from the metallic copper standard (Figure 50).

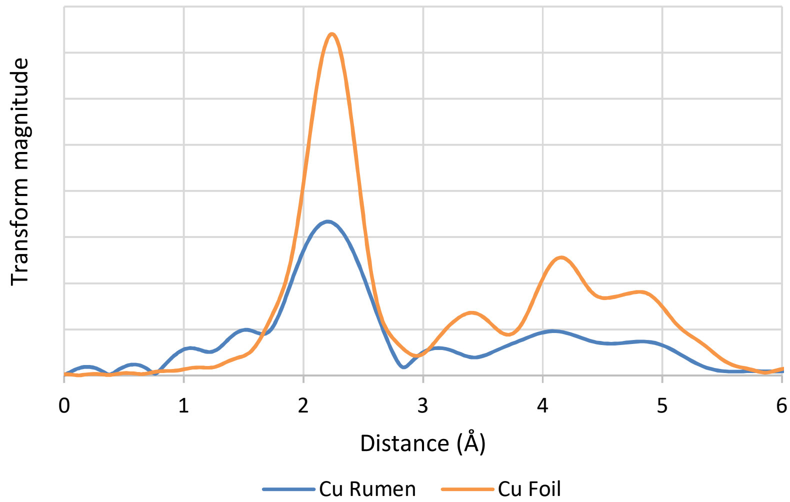

Figure 50: Copper K-edge data plotted in R-space for rumen sample and metallic copper standard.

The similarity of distance in shells at 2.2, 4.0 and 4.9 Å, although different in magnitude support the XAFS model for Cu-Cu bonds. Additionally, the closeness of E^0 previously discussed (Section 7.4.1) for both rumen and metallic copper suggests that metallic copper may be a contributing compound in the rumen digesta. Principle component analysis (PCA) was conducted on the rumen data set against the standard data from bornite, chalcopyrite, copper sulphate and the copper foil to determine the likelihood of the standards contributing to the heterogeneous sample. The results indicated that contributions by copper sulphate and chalcopyrite were low and that contributions from bornite were possible and a contribution from metallic copper was very likely; showing a high correlation. Linear combination fitting (LCF) confirmed these observations indicating that copper sulphate and chalcopyrite contributed ~12% (±11) and 0% (±7) to the mixture respectively. Bornite and metallic copper contributed 57% (±8) and 31% (±4) respectively (R= 0.007). A major limitation of the LCF model is that it assumes the standards fitted in the model will describe the sample to 100%. The use of PCA determines the likelihood of contribution. However, if likely candidates are included alongside others which are unlikely to contribute, or the only available candidates are unable to describe the compound in its entirety the contributing percentage ascribed to the correlating factor is falsely elevated. In this situation, where the model cannot be fully described with the available candidates it suggests ~30% contribution of metallic copper in this compound, but the actual contribution may be vastly lower if better describing candidate compounds were available. To test this, the ovine liver data collected in Chapter 6 was computed into the linear combination fit, to represent a biological copper-sulphur containing compound; although it is unlikely that this compound represents a meaningful candidate in reality. The inclusion of this candidate reduced the estimated percentage of metallic copper to 6% (±4) and gave a 91% (±14) contribution from the liver data (R= 0.003), demonstrating a more realistic contribution from metallic copper and emphasising the distortion effects of the model. Further modelling with more realistic candidates would be able to further refine this model and provide an accurate contribution for Cu^0.

The first shell of the jejunum sample was fitted with a single scattering oxygen pathway ~1.9 Å followed by three co-ordinated sulphur single scattering pathways at ~2.3 Å, consistent with the XANES observations of tetrahedrally co-ordinated copper. The further shells were less easily defined in this model and were best represented by a single scattering chlorine pathway at ~3 Å (n=2) followed by a double scattering pathway comprising sulphur and iron at ~4 Å (n=4), a further single scattering sulphur pathway at ~4.3 Å (n=3) and lastly a single scattering iron pathway (n=1) at ~4.5 Å. Figure 51 shows the normalised and fitted paths. All fitted paths and distances are tabulated in Table 14.

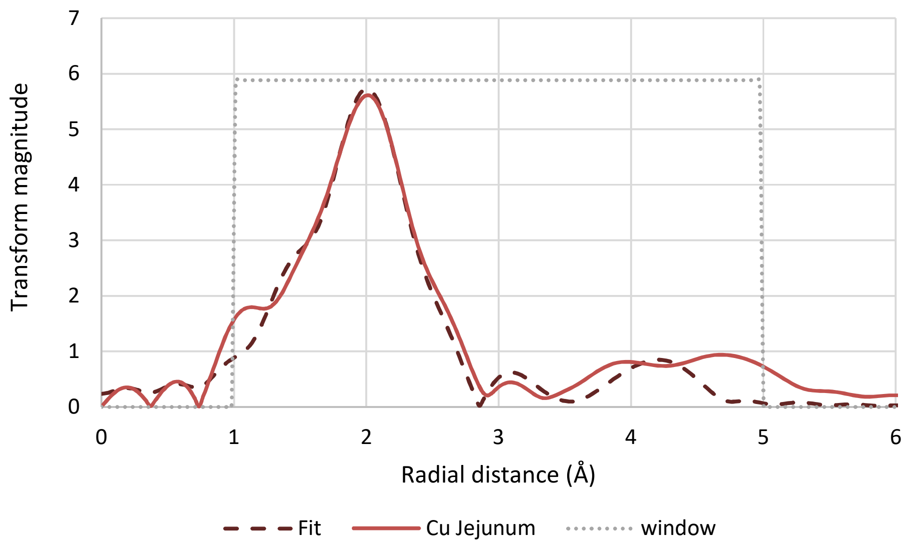

Figure 51: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for copper K-edge jejunum digesta. Solid line denotes sample. Dashed line denotes peak fit. Dotted line denotes window for peak fitting used in model.

Table 14: n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ^2(Å^2) for best fit data for jejunum copper K-edge. R-factor indicates goodness of fit (<0.02).

				Co-ordination shell				
Sample	**Path**	**n**	S_0^2	**R(Å)**	**σ^2(Å^2)**	E_0	**Reduced χ^2**	**R-factor**
Jejunum	Cu-O	1	1.05	1.899	0.012 (±0.011)	6.06	1707	0.019
	Cu-S	3	-	2.318	0.013 (±0.005)	-	-	-
	Cu-Cl	2	-	2.914	0.008 (±0.002)	-	-	-
	Cu-S-Fe	4	-	4.102	0.0002 (±0.012)	-	-	-
	Cu-S	3	-	4.371	0.023 (±0.024)	-	-	-
	Cu-Fe	1	-	4.510	0.006 (±0.007)	-	-	-

PCA was also conducted on the jejunum data set against the copper standards. The results indicated that contributions by copper sulphate and chalcopyrite were unlikely, a contribution from bornite was likely, and a contribution from metallic copper was also possible. LCF confirmed these observations. Chalcopyrite contributed 0% (±4) and copper sulphate 13% (±7). Whereas metallic copper contributed 16% (±2) and bornite 71% (±5) to the model (R= 0.002).

7.4.3.2 XAFS of the iron K-edge

The XAFS spectra for both rumen and jejunum samples at the iron K-edge look superficially different but appear to have similarity with respect to their peak distance. There is similarity in peak distance at 1.5, 2.3, 2.8, 3.4 and 4.4 Å with a similar magnitude at 3.4 Å too. Suggesting that the contributing atoms may be similar but with slightly different bond lengths and co-ordination numbers. Figure 52 shows the XAFS spectra for rumen and jejunum samples at the iron K-edge.

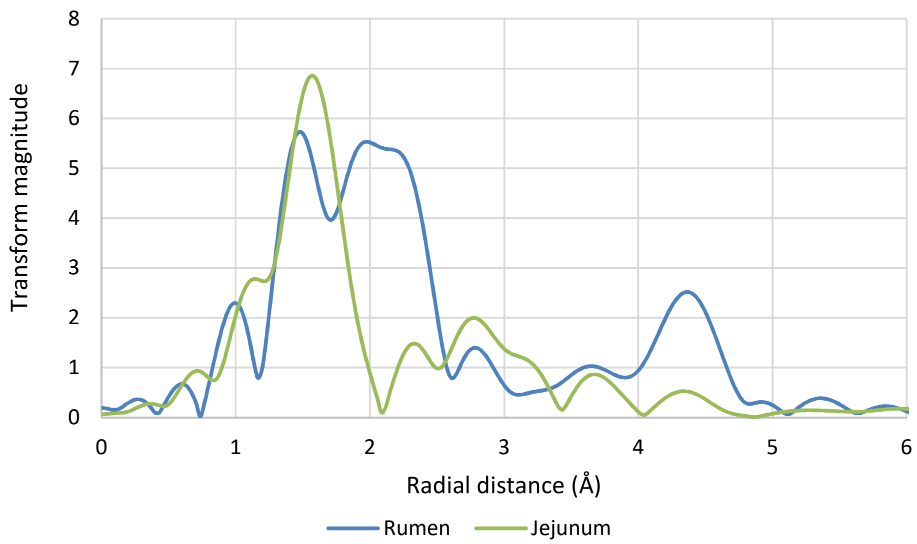

Figure 52: XAFS spectra plotted in R space for K^2 weighted rumen and jejunum sample data.

The data were both fitted with single scattering oxygen at 1.2 Å (n=3) to fit the initial peak. The XANES spectra for these samples was indicative of tetrahedral, trigonal bi-pyramidal or square pyramidal. Although, increasing the co-ordination to 4 or 5 in these samples reduced the quality of the fit to the initial peak. The next peaks created good fits with single scattering sulphur atoms. The atoms varied in their radial distance and co-ordination number to allow the peak distance and magnitude to be plotted. Figure 53 shows the normalised and fitted paths. All fitted paths and distances are tabulated in Table 15.

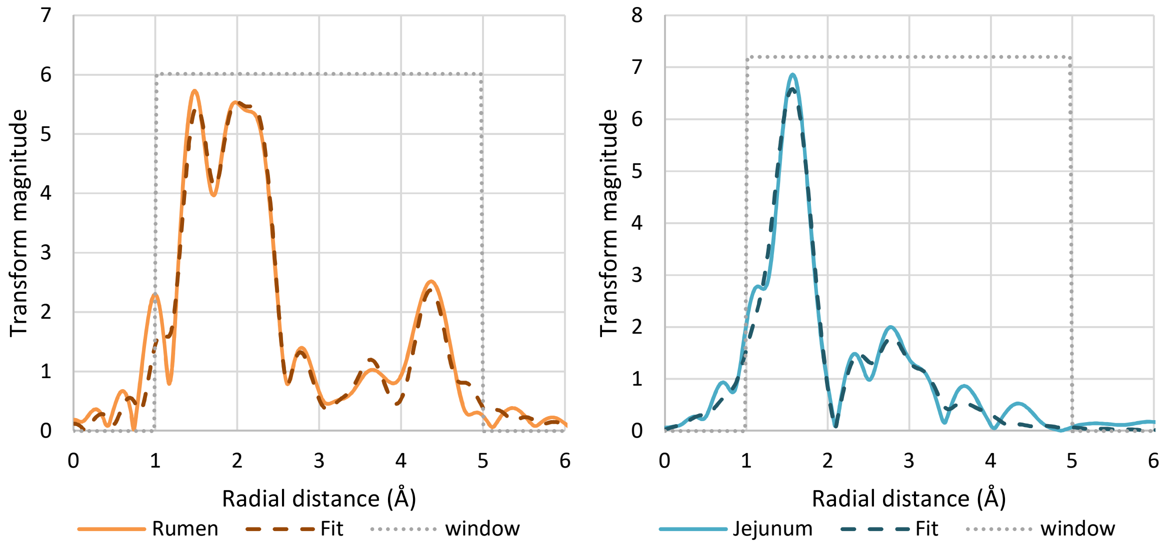

Figure 53: XAFS peak fitting to Fourier transformed data plotted by magnitude in R-space for rumen and jejunum digestive samples at the iron K-edge. Solid line denotes sample. Dashed line denotes peak fit. Dotted line denotes window for peak fitting used in model.

After 3 Å the samples spectra shares the most similarity, notwithstanding magnitude in some peaks. These peaks were fitted with an oxygen-sulphur obtuse triangle scattering pathway, a single scattering sulphur pathway and an obtuse triangle sulphur-iron scattering pathway respectively, using differing co-ordination to replicate magnitude.

Table 15: n co-ordination number, interatomic distances R(Å) and Debeye-Waller factors σ^2(Å^2) for best fit data for rumen and jejunum iron K-edge digestive samples. R-factor indicates goodness of fit (<0.02).

					Co-ordination shell			
Sample	**Path**	**n**	S_0^2	**R(Å)**	**σ^2(Å^2)**	E_0	**Reduced χ^2**	**R-factor**
Rumen	Fe-O	3	0.9	1.997	0.006 (±0.002)	1.86	2423	0.01
	Fe-S	1	-	2.444	0.000 (±0.001)	-	-	-
	Fe-S	2	-	2.636	0.006 (±0.003)	-	-	-
	Fe-O-S	2	-	3.351	0.001 (±0.007)	-	-	-
	Fe-S	8	-	4.043	0.029 (±0.001)	-	-	-
	Fe-S-Fe	8	-	4.776	0.001 (±0.003)	-	-	-
Jejunum	Fe-O	3	1.15	1.997	0.007 (±0.003)	-1.01	743	0.01
	Fe-S	1	-	2.444	0.009 (±0.005)	-	-	-
	Fe-S	4	-	2.636	0.051 (±0.023)	-	-	-
	Fe-O-S	4	-	3.351	0.005 (±0.003)	-	-	-
	Fe-S	8	-	4.043	0.04 (±0.011)	-	-	-
	Fe-S-Fe	4	-	4.776	0.046 (±0.106)	-	-	-

PCA was conducted on the rumen and jejunum data sets against iron standards; bornite, chalcopyrite, iron sulphate and metallic iron. The rumen results inducted that a contribution by chalcopyrite was highly unlikely and that contributions from bornite were possible while contributions from both metallic iron and iron sulphate were probable; with high correlations. LCF confirmed chalcopyrite contributed 0% (±12) and bornite 32% (±13). Whereas metallic iron and iron sulphate contributed 25% (±6) and 43% (±19) to the model respectively (R= 0.012). The principle component analysis for the jejunum data differed to the rumen sample. Suggesting a lower likelihood of a contribution from metallic iron in this sample and only a high correlation to iron sulphate. The LCF reported chalcopyrite constituted 0% (±16), metallic iron contributed 8% (±7).While bornite contributed 34% (±18) and the most contribution came from iron sulphate with 58% (±25) (R= 0.019).

7.5 Discussion

The XAS data provided evidence that copper present in both digestive regions had a high likelihood of being present as Cu^{+} with some contribution from Cu^{0}; especially in the rumen. The transition from Cu^{2+} into Cu^{+} can be explained through the low redox potential (defined as <100 mV, but often negative) of the rumen (described in Section 1.5). This environment is sufficient to promote the reduction of Cu^{2+} into Cu^{+} using a similar mechanism to the intracellular environment; which is known to create an environment more conducive to Cu^{+} than Cu^{2+} (Helsel and Franz, 2015; Johnson *et al.*, 2015) through the maintenance of a cytoplasmic redox potential between -240 and -290 mV (López-Mirabal and Winther, 2008). At -350 mV (Kamra, 2005), the rumen, although extracellular, creates a strong reducing environment able to promote the spontaneous reduction of dietary Cu^{2+} into Cu^{+} in a similar way. Thermodynamically, the spontaneous reduction of Cu^{2+} into Cu^{+} is supported where the concentration of Cu^{+} is lowered through complexation with the abundant rumen ligands; consequently lowering the ratio of $Cu^{+}:Cu^{2+}$. Subsequently, Cu^{2+} is reduced to Cu^{+} to re-establish the original ratio valid at the redox potential (Leal and Van Den Berg, 1998).

Interestingly, the oxidation state of rumen copper was surprisingly close to metallic copper, only 1 eV above the Cu^{0} E^{0}. Photo-reduction can occur in samples where the beam causes radiation damage to the sample reducing the E^{0} (Gonçalves *et al.*, 2013). However, in this experiment the sample position was moved between each scan to minimise this effect and both the XAFS peak fitting model and XANES spectra demonstrated some resemblance to metallic copper. The results of the PCA and LCF also suggested that there was a component of metallic copper present in the heterogeneous rumen sample, despite the limitations of the model preventing an accurate percentage contribution from being obtained.

It is feasible to cycle reversibly between Cu^{0} and Cu^{+} by exposing nanoparticles (<25 nm) to oxidising conditions; such as exposure to air, and reducing conditions; such as those in the rumen environment (Pike *et al.*, 2017). Within the rumen there are several strong reducing agents with the potential to elicit an electron exchange into Cu^{0} from Cu^{+} including; Fe^{2+} compounds, sulphite (SO_3^{2-}) containing compounds, sugars which contain a free aldehyde or ketone group (CHO or CO respectively) and even ascorbic acid (vitamin C), each of which in combination with the low redox potential of the rumen could further reduce copper as Cu^{+} into Cu^{0}. In addition to a low redox potential, unbound Cu^{+} in aqueous solution is able to disproportionate; a reaction where the intermediate ion is able to both oxidise and reduce itself. In this case, Cu^{+} would disproportionate into Cu^{2+} and Cu^{0} following the equation; $2Cu^{+}_{(aq)} \rightleftharpoons Cu^{2+}_{(aq)} + Cu^{0}_{(s)}$ (Johnson *et al.*, 2015). If the reducing nature of the rumen promotes formation of aqueous Cu^{+}. A proportion of the resulting Cu^{+} could become bound to competing rumen ligands. Since disproportionation can only occur with simple Cu^{+} complexes in solution, complexation with ligands, other than water, will stabilise the copper in its Cu^{+} state (Clark 2015). The remaining proportion of Cu^{+} could potentially disproportionate, giving rise to some Cu^{0} which is stable, and some Cu^{2+} which is instantly reduced into Cu^{+} again by the rumen environment to be subject to further competition. The presence of metallic copper poses a problem for the rumen environment. The microbiota which are present there are fundamentally ill-equipped to handle copper (discussed in Section 9.3.3), and the anti-microbial properties of metallic copper are well documented (Espírito-Santo *et al.*, 2011; Mathews *et al.*, 2013; Wang *et al.*, 2017), although the mode of action is reliant on direct surface contact with the bacteria and is inhibited in solution (Espírito-Santo *et al.*, 2011; Mathews *et al.*, 2013). Thus, it is possible that a small percentage of the rumen copper could be present as nano-particulate metallic copper. The high mathematical contribution from ovine liver data in the LCF model highlights that the contribution from metallic copper is likely to be very small in reality (<10%) and could successfully co-exist with the rumen micro-organisms.

The evidence gathered in this study indicates that the digestive copper compounds differ notably from the fed copper source which was Cu^{2+} as copper chloride ($CuCl_2 \cdot 2H_2O$). Evidence from the E^{0}, XAFS and XANES spectra all support the notion that this copper source was subject to dissociation within the rumen and no proportion of copper chloride remained. Previous work has used computer modelling to estimate the likelihood of Cu^{2+} speciation in the bovine rumen from; copper chloride, copper glycine, copper lysine and copper methionine, and found that 60% of copper regardless of initial source was likely to be present as carbonate species, followed by; phosphate (24%), acetate (10%), other volatile acids (4%), ammonia (1%) and aquated copper (0.2%) (Essilfie-Dughan, 2007). The present data agrees that speciation of copper source takes place, but in the present trial did not create a viable XAFS peak fit with Cu^{+} carbonate. The XANES spectra were also very different between the rumen sample data and reference spectra from copper carbonate, copper phosphate, copper acetate, and copper citrate indicating that these were not the predominant species of copper present in the rumen. The previous trial used computer modelling in combination with some simulated rumen fluid work to generate their data. It is likely that this model was insufficient to fully replicate the rumen environment and as such may have led to an overestimation of the contribution of salivary complexes (carbonate and phosphate); explaining the absence of these compounds in the *in vivo* data presented here.

The XANES analysis indicated that the rumen compound was likely to be 3 or 4 co-ordinated which was corroborated by the XAFS peak fit which created a good fit with four co-ordinated sulphur atoms in the first shell. The resemblance of XANES spectral shape to bornite, ovine liver, cysteine and Cu^{+} sulphide, all of which comprise Cu^{+} 3 or 4 co-ordinated to sulphur, strongly suggest that the majority of copper in the rumen may be in a Cu^{+} thiol complex. The fit created with tetrahedral sulphur co-ordination in the first shell is inconsistent with metallic copper and the spectra plotted in both energy and R space indicate that at least one other copper containing compound is present. A Cu^{+} thiolate compound is supported by synergistic bonding creating a stable complex (Rizvi *et al.*, 2015). Notably, Cu^{2+} thiolate compounds are known to be unstable in solution and within the rumen could be reduced into Cu^{+}, whereas Cu^{+} sulphur compounds which are either formed or fed are particularly effective at stabilising copper. Using the HSAB theory (discussed in Section 6.5), the soft-soft interaction of Cu^{+} and sulphur will occur more rapidly and form a stronger bond than $Cu^{2+}S$. This principle is also seen in biological thiol complexes as Cu^{2+} complexes with glutathione and cysteine are much less stable than Cu^{+} complexes relating to the soft Lewis acid character of Cu^{+} (Le Gall and Van den Berg, 1993). Glutathione is known to reduce Cu^{2+} to Cu^{+} during complex formation (Leal and Van Den Berg, 1998), this suggests that in Cu^{+}-thiol complexes, Cu^{2+} is first reduced to Cu^{+} in the presence of the thiol, and then complexes, as a result of the specific complexation of Cu^+ (Leal and Van Den Berg, 1998).

Beyond the pre-edge and edge regions the rumen and jejunum copper spectra were very different. The XAFS peak fitting indicated that there were substantial changes in copper speciation between the two regions of the digestive tract. The copper in the jejunum also shared the greatest resemblance in XANES spectra to bornite, cysteine and Cu^+ sulphide, indicative of a 3 or 4 co-ordinated compound. This compound was successfully XAFS peak fitted with an oxygen-sulphur tetrahedron in its first shell and further shells fitted with chlorine, iron and sulphur. The combination of both iron and sulphur in this fit provide the first insight of a compound containing the elements in the copper-iron-sulphur antagonist pathway. The PCA and LCF showed the greatest similarity to bornite; a copper, iron, sulphur complex known in nature.

Bornite an insoluble, copper-iron-sulphide with the chemical formula Cu_5FeS_4. Its crystalline shape is orthorhombic at biological temperatures with four sulphur atoms, five copper atoms and one iron atom making up the cubic unit. The first copper atom is tetrahedrally co-ordinated with sulphur with copper in its Cu^+ oxidation state and iron as Fe^{2+} (Goh *et al.*, 2006; Zhao *et al.*, 2014). While the jejunum compound shares the tetrahedral co-ordination, in the present model it comprised three sulphur and one oxygen atoms. Due to the partial occupancy of the bornite shell structure, it was not possible to create and XAFS peak fit using bornite. Preventing the similarity of local geometry and co-ordination between these two compounds from being established. However, it is not expected that enough similarity exists between the two compounds for the peak fit to be valid. It appears that only a similarity exists between these two compounds, but perhaps gives a first insight into a similar structure worthy of further investigation for this antagonist compound.

The XAS data indicated that the effects of the rumen as a reducing environment also affected the oxidation state of iron. Although, this appeared to a lesser extent, as a proportion of the iron present was still likely to be Fe^{3+} in the heterogeneous sample.

The XANES and XAFS spectra were very similar for both digestive regions, differing only in peak magnitude. Suggesting that although some of the compounds may undergo changes the majority of the iron is consistently complexed as it moves through the digestive regions. The spectra and E^0 of the sample data were very different from that of the fed compound, in agreement with the copper data, that the speciation of soluble iron compounds also takes place within the rumen. A comparison of the XAFS and XANES data suggested that the iron compound was tetrahedrally co-ordinated by three oxygen and one sulphur atom and bore the most resemblance to Fe^{2+} sulphide and Fe^{3+} sulphate, potentially suggesting a mixture involving both species. The abundance of sulphur in the XAFS fit for the further shells could explain the similarity between these spectra. In addition to iron sulphate there was also some resemblance to bornite in the LCF, although the potential contribution by iron sulphate was much greater. Unfortunately, no copper atoms could be successfully fitted into the iron XAFS peak fit. The absence of copper could be explained by a much lesser proportion of iron being involved in the copper-iron-sulphur pathway; perhaps in a similar ratio to that observed in bornite of 5:1 Cu:Fe. Since iron is generally present in a greater concentration than copper in digestion it is possible that the proportion of iron interacting in the copper-sulphur moiety is too small to impact the overall spectra of this element. The two main modes of action suggested for this pathway (discussed in Section 1.8) suggest that iron sulphide may displace with copper sulphide or that a copper iron sulphide forms. Both of these modes of action are still possible based on the evidence from this trial. If a compound similar to copper-iron sulphide is forming it has been established to be closer in structure to bornite than chalcopyrite. The elimination of direct sulphide formation cannot be ruled out, the similarity of spectra and number of fits which were enhanced through thiol and sulphur groups show the effects of this abundant ligand in the rumen. The indications from this trial suggest that copper complexation with iron and sulphur may occur beyond the rumen and may involve a more complex compound involving oxygen and chlorine in addition to copper sulphur and iron.

7.6 Conclusion

The reducing environment created in the rumen is capable of reducing both copper and iron into Cu^{+}, Cu^{0} and Fe^{2+}. The oxidised forms of these metals fed in a soluble ionic source showed complete dissociation from the initial source in the rumen. The copper complex changes dramatically as it progresses through digestion into the small intestine. Whereas the majority of the iron, likely complexed to sulphur ligands, remains in a very similar compound through both of these regions. The copper edge of the jejunum sample gave the first ever reported indication of the formation of a complex involving copper, iron and sulphur. Although, present in further shells and therefore harder to define, this compound may be more complex than the original hypothesis suggest.

8.0 Testing copper sources against an antagonist challenge *in vivo*

8.1 Introduction

The work conducted in Chapters 3 and 4, alongside the evidence from other workers discussed in these chapters, highlights the variety of behaviour across different copper sources during digestion. These differences may lead to different participation in the interactions that inhibit copper absorption, making some copper sources more bioavailable than others in the presence of antagonists. Until now, the partitioning of different copper sources through digestion has not been studied and the ability of different copper sources to detoxify an antagonist problem in comparison to each other has not been determined.

8.2 Aims & Objectives

The aim of this work was to trace the movement and solubility of different copper sources through digestion under an antagonist challenge from; molybdenum, iron and sulphur. To observe any differences and to determine the percentage of copper which remains potentially bioavailable in the faeces.

8.3 Materials & Methods

8.3.1 Experimental design

Four 6 year old female Holstein-Friesian cows (BW $\bar{x}$ 832±25 kg) with rumen fistulae were housed on tie-stall cubicles for 4 weeks. Cubicles were furnished with rubber mattresses and cleaned at least twice daily. Ventilation, daylight and temperature were all controlled to ambient conditions. Experimental work was conducted in the Netherlands under DEC (Dutch Ethics Committee) approval number 20400 and UK ASPA (Animals (Scientific Procedures) Act 1986) non-UK 00010 approval.

Before being introduced to the cubicles animals received the experimental basal diet comprising 2 kg/day formulated compound feed (Table 16) and 10 kg/day (±2 kg) short-chopped wheat straw for a period of 4 days prior to the trial start date.

Table 16: Compound feed ingredients and percentage inclusion

Ingredient	Maize	Soyabean meal	Cane molasses	Salt (NaCl)	Salt (CaP)	Salt (MgO_2)	Salt (Na_2SO_4)
Percentage	38	51.63	5	2	1.7	0.55	1.12

Straw ration was calculated and offered at 100% of previous straw intake averaged over a fortnight. The experimental basal diet was fed throughout the trial (Table 17). Compound feed was first provided each morning and once eaten, straw provided as forage in two 5 kg meals. Leftover straw was weighed and recorded. Waste straw from the floor was also collected and weighed to account for loss in the ration. Animals were fed from 7:30 am each morning. Allowing a 10 minute interval between animals for sampling. Once they had received their compound feed their allocated treatment was placed directly into the rumen fistula every morning for 4 days. The final diet comprised the basal diet plus a treatment which provided copper at a final inclusion of 16 mg/kg DM and an antagonist challenge from both iron and molybdenum (Table 17).

Table 17: Mineral content of the basal diet for controlled elements as analysed by ICP-MS ± standard deviation.

	Iron mg/kg DM	Molybdenum mg/kg DM	Copper mg/kg DM	Sulphur g/kg DM	% of total diet
Wheat straw	44±1	0.5±0.2	2.4±0.2	1.4±0.04	83.3
Compound feed	346±3.7	2.1±0.03	8±0.5	17±0.02	16.7
Concentration in complete basal diet	**94.3**	**0.8**	**3.3**	**4**	**100**
Concentration in final diet (including treatment)	**150**	**10**	**16**	**4**	

Treatments were different by copper source. Each treatment contained one of; 'CuEDTA' (Copper EDTA, Sigma-Aldrich, Dorset, UK. ≥97.0%), 'CuO' (copper oxide, Fisher Scientific, Loughborough, UK. >99%), 'CuCl' (copper chloride, VWR International Ltd, Lutterworth, UK ≥99.0%) or 'TBCC' (Tribasic copper chloride, Intellibond®, Selko®, Tilburg, NL). Treatments were prepared into 250 g polypropylene bottles containing a powdered mixture of copper, sodium molybdate (Fisher Scientific, Loughborough, UK) and iron chloride (Fisher Scientific, Loughborough, UK) calculated to the final inclusion rates shown in Table 17, alongside the solid and liquid phase markers; 45 g/day titanium dioxide (VWR International Ltd, Lutterworth, UK) and 20 g/day polyethylene glycol (PEG mw 6,000, Sigma-Aldrich, Dorset, UK) as binders. Animals were randomly allocated to a treatment group using MS Excel (2013, Microsoft, Washington, USA). Treatments were allocated to random blocking and colour coded for blinding purposes. The treatment groups conformed to a 4x4 Latin square design.

On day 1 and 4 samples were collected at 1, 3, 5 and 7 h post-treatment (Figure 54). Animals had 3 rest days where basal diet was provided without treatment prior to the start of the next trial period.

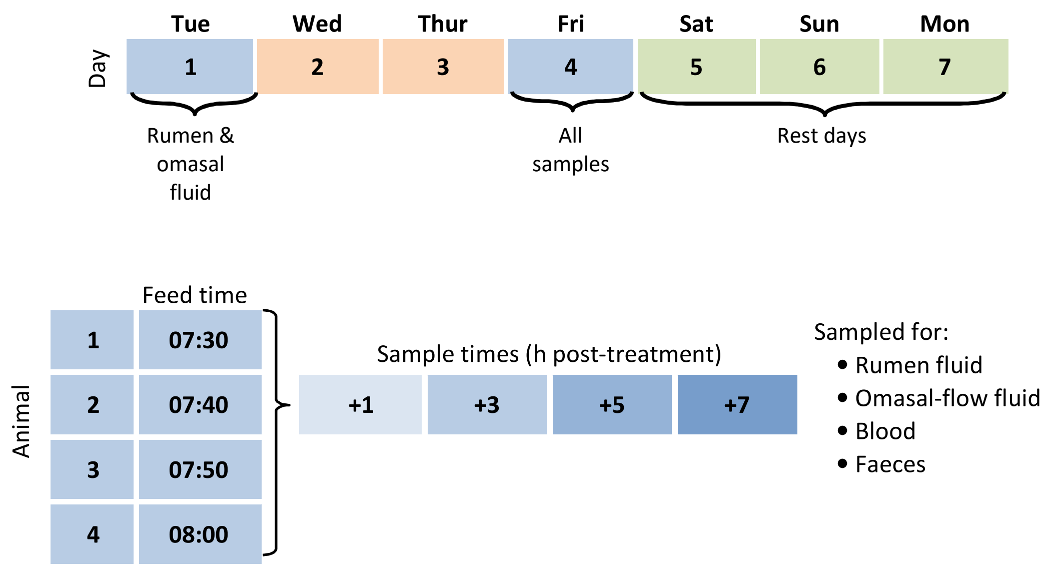

Figure 54: Outline of trial period, sample and rest days and time points used for feeding and sampling

8.3.2 Sample collection and analysis

Rumen and omasal-flow fluid was collected on day 1 and day 4 of each experimental period using the methods outlined in Section 5.3.2.2. Day 1 represented the novel adaptation of the rumen to each treatment and day 4, the steady state. Faeces and blood were collected only on day 4 of each experimental period using methods from Sections 5.3.2.1 and 5.3.2.3. Samples were subject to the same preparation and analysis as described in Section 5.3.3.

In this trial, blood was collected into 2 x 10 ml LH and 2 x 10 ml serum vacuum tubes containing silicone clotting activator (CAT, Vacutainer®, Becton-Dickinson, Winnersh, UK), using the method described in Section 5.3.2.3. To measure haematocrit, prior to centrifugation or freezing LH tubes were mixed for 10 minutes on a tube roller (SciQuip, Shropshire, UK). Blood was then drawn into capillary tubes (75 mm, non-heparin, Hirschmann, Eberstadt, Germany), stoppered (Brand®, Sigma-Aldrich, Dorset, UK) and centrifuged at 13,000 *g* for 5 mins (acceleration 0-11,800 rpm in 70 s, Haematospin 1300, Hawksley, Sussex, UK). Haematocrit was manually measured using a ruler and calculated as a percentage of cellular content to plasma fraction. All other blood analysis was conducted using the previous methods described in Sections 5.3.3.4-5.3.3.7.

8.3.3 Digestive marker analysis

Digestive marker analysis for titanium dioxide and PEG were conducted using the methods described in Section 5.3.4.

8.3.4 Data analysis

Data for this study was analysed using the methods described in Section 5.3.5, and previously determined levels of significance.

8.4 Results

8.4.1 Digestive parameters

The rumen fluid (as both supernatant and colloid as defined in Section 5.3.3.1) showed a peak in copper concentration at 1 h following the treatment dose which continued to decline over time (Figure 55), in a similar pattern to that observed in the previous *in vivo* trial (Chapter 5). Sample day 2 (steady state) reported a ($P<0.01$) higher copper concentration in the rumen colloid than sample day 1 (novel introduction), but there was no significant effect of sample day on the rumen supernatant despite the values for day 2 being numerically higher (data not shown). No treatment x time interactions were observed in this data.

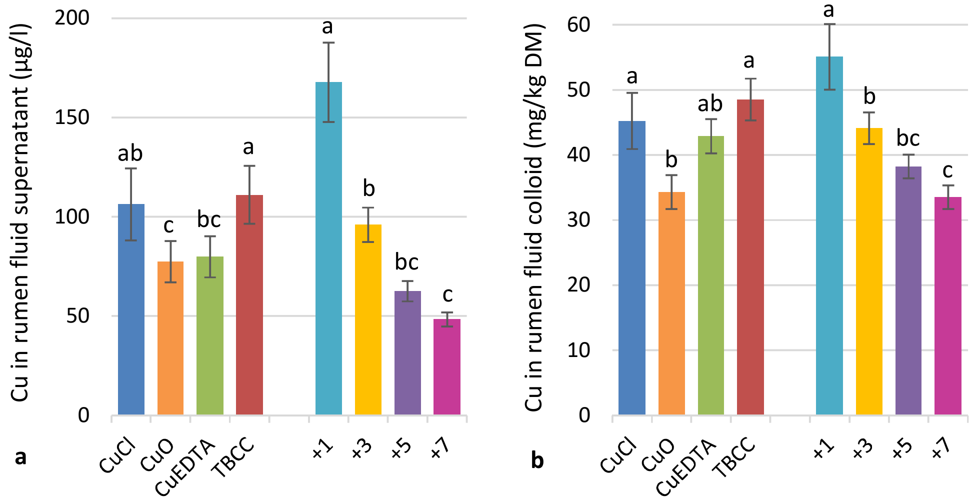

Figure 55: Copper concentration in the rumen supernatant (a) and colloid (b) by treatment and time. Error bars denote standard error of the mean. Different letters indicate significant difference by treatment or by time.

In the rumen fluid supernatant (Figure 55a), there was ($P<0.05$) more copper from tribasic copper chloride (TBCC, 111±82 µg/l) and copper chloride (CuCl, 106±103 µg/l) than copper EDTA (CuEDTA, 80±59 µg/l) and copper oxide (CuO, 77±59 µg/l). Within the colloidal fraction (Figure 55b) there was also a ($P<0.01$) higher concentration of copper from TBCC (48±18 mg/kg DM), than CuO (34±15 mg/kg DM). Although, the other copper sources were not significantly different to TBCC (x̄ 44±20 mg/kg DM, CuCl & CuEDTA combined). The percentage of total copper in the supernatant fraction remained ~20% (23±9%), similar to the results in Chapter 5, with no difference between the copper sources, and the majority of copper irrespective of source associated with the colloidal fraction.

Iron concentration in the supernatant fraction of the rumen fluid (Figure 56a) was ($P<0.0001$) higher with CuEDTA as a copper source (656±272 µg/l) than the other copper sources. No difference was detected between the other copper sources (x̄ 446±158 µg/l, CuCl, CuO & TBCC). Iron in the colloid had no significant difference for treatment and remained at a mean of 245 (±51) mg/kg DM (Figure 56b). Around 80% of the iron was associated with the supernatant in the rumen fluid, however, ($P<0.0001$) less (72±10%) was associated here for CuEDTA. Iron concentration was also ($P<0.01$) different by sample day for both rumen supernatant and colloid. For both fractions of rumen fluid, the iron concentration was higher on the steady state sample day (data not shown). Only the colloidal fraction reported an (P<0.0001) effect of time. No treatment x time interaction was observed.

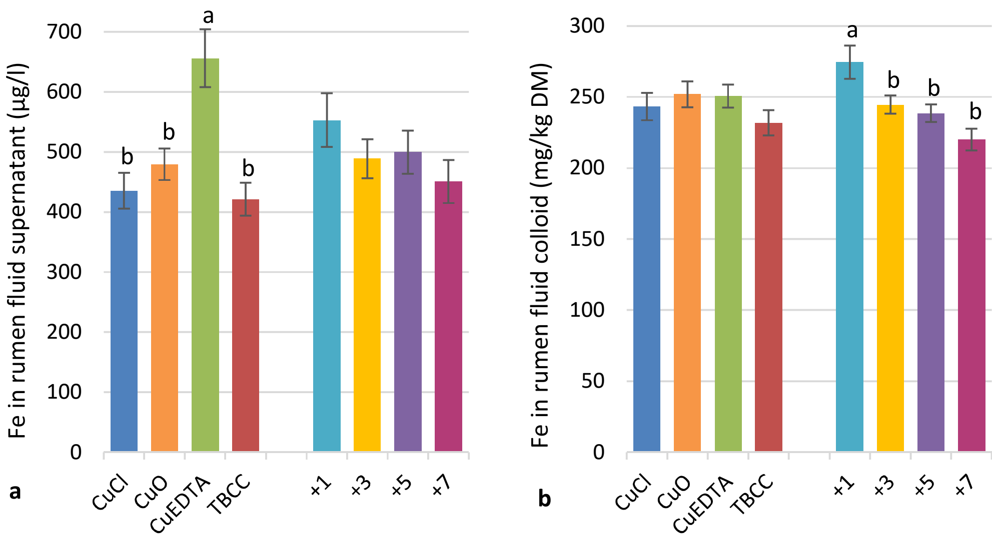

Figure 56: Iron concentration in the rumen supernatant (a) and colloid (b) by treatment and time. Error bars denote standard error of the mean. Different letters indicate significant difference by treatment or by time.

Molybdenum concentration was not significantly different between the copper sources in rumen supernatant (x̄ 86±106 µg/l) or colloid (x̄ 21±12 mg/kg DM) (Figure 57). Around 30% of the molybdenum was associated with the supernatant in the rumen fluid; which was around twice as high as the Chapter 5 data (Section 5.4.1). There was a (P<0.001) higher percentage of molybdenum in the supernatant where CuO (36±15%) was used as the copper source compared to CuEDTA (27±13%). In a similar pattern to the other elements studied, molybdenum also reported higher (P<0.001) concentrations on the steady state sample day for both the rumen supernatant and colloid and the peak and subsequent decline in concentration through the time points was present (P<0.0001) (Figure 57), no treatment x time interactions were reported.

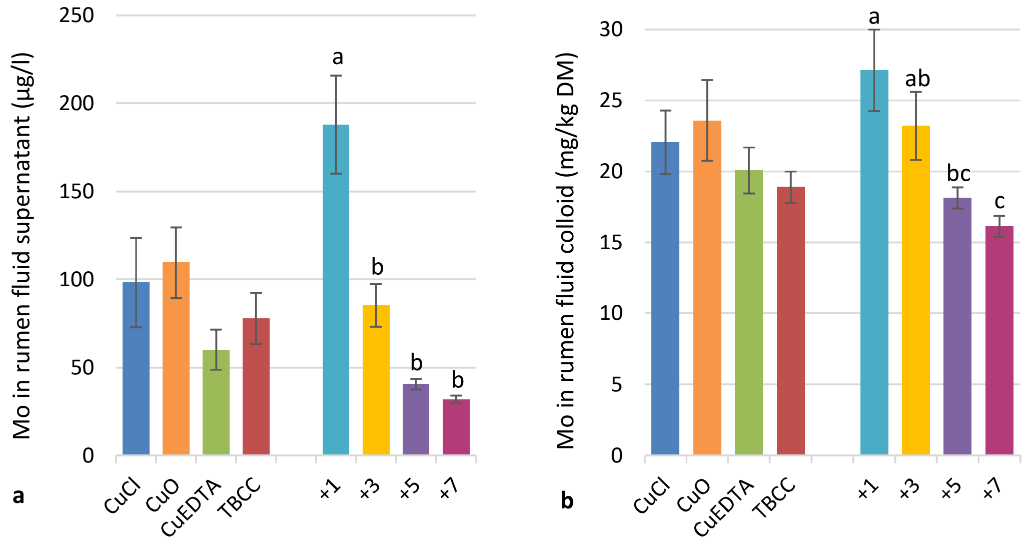

Figure 57: Molybdenum concentration in the rumen supernatant (a) and colloid (b) by treatment and time. Error bars denote standard error of the mean. Different letters indicate significant difference by treatment or by time.

The copper concentration of the omasal-flow supernatant and colloid across all treatments had copper concentrations lower ($P<0.05$) in the first period than the subsequent periods. Similar to the effect observed in Chapter 5, copper appeared to accumulate in the omasal-flow. The data in this trial showed that this accumulation was still highly variable with high standard error and standard deviations. In comparison to their respective fractions of rumen fluid, more ($P<0.01$) copper was present in the supernatant and ($P<0.001$) colloidal fractions of the omasal-flow; equating to between 10-60 times more copper in the omasal-flow than rumen fluid. This effect was greatest for TBCC which was 60 times higher, followed by CuO at 33 times higher, CuEDTA at 15 times higher and lastly CuCl at 10 times higher.

In the omasal-flow supernatant ($P<0.05$) more copper was present with TBCC (6,707±17,678 µg/l) than CuEDTA and CuCl (1,167±3,139 & 1,110±2,070 µg/l respectively). Interestingly, CuO had the second highest copper concentration in the omasal supernatant (2,519±5,248 µg/l) (Figure 58a).

The omasal-flow colloid had ($P<0.01$) more copper from TBCC (1,520±3,362 mg/kg DM), than the other copper sources (range 236-484 mg/kg DM) (Figure 58b). The percentage of total copper which remained in the supernatant was ($P<0.01$) higher for TBCC (31±21%) in comparison to CuEDTA (19±22%); which had the least in the soluble fraction. The novel sample day showed no difference between the copper sources for the copper content of omasal-flow supernatant, colloid or percentage in the soluble fraction. On the steady state sample day, the omasal colloid had ($P<0.05$) more copper from TBCC (2,071±4,465 mg/kg DM) compared to CuEDTA (102±57 mg/kg DM) and the omasal-flow supernatant also had ($P<0.05$) more copper from TBCC (1,1644±24,201 µg/l) than CuCl and CuEDTA (948±2,487 & 192±224 respectively).

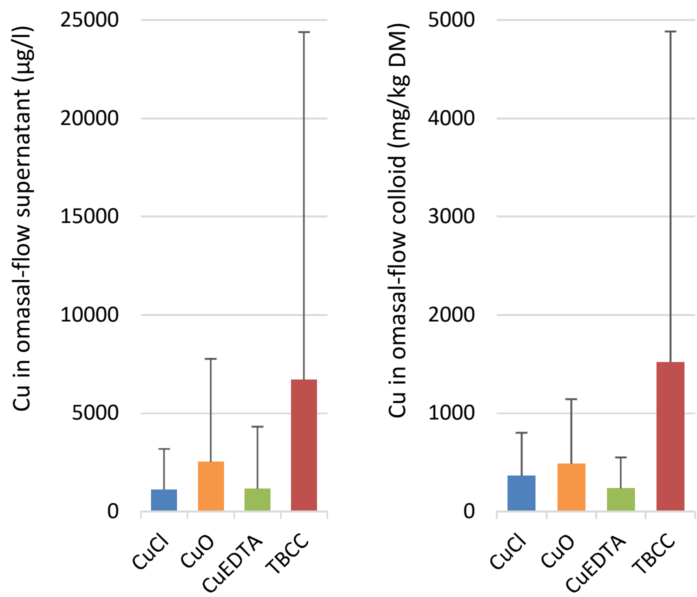

Figure 58: Copper concentration in omasal-flow supernatant (a) and colloid (b) by treatment. Error bars denote standard error of the mean. Different letters indicate significant difference by treatment.

Across both sample days, numerically more iron was present in the omasal-flow supernatant for CuEDTA (1,072±693 µg/l), but this was not significantly greater than the other copper sources ($\bar{x}$ 976±634 µg/l combined mean) (Figure 59a). Around twice as much iron ($P<0.0001$) had accumulated in the omasal-flow supernatant than in the rumen fluid. There were no significant differences in iron content of the colloid for the different copper sources ($\bar{x}$ 393±285 mg/kg DM combined mean) (Figure 59b). However, a higher ($P<0.01$) percentage of iron (75±14%) was associated with the supernatant for CuEDTA than CuCl (67±22%) in the omasal-flow. There were no significant differences between the novel sample day and steady state data for iron content of the omasal-flow supernatant or colloid. However, on the novel day there was a (P<0.0001) higher iron percentage in the supernatant for CuEDTA (77±17%) and TBCC (76±14%) sources in comparison to CuCl (63±23%).

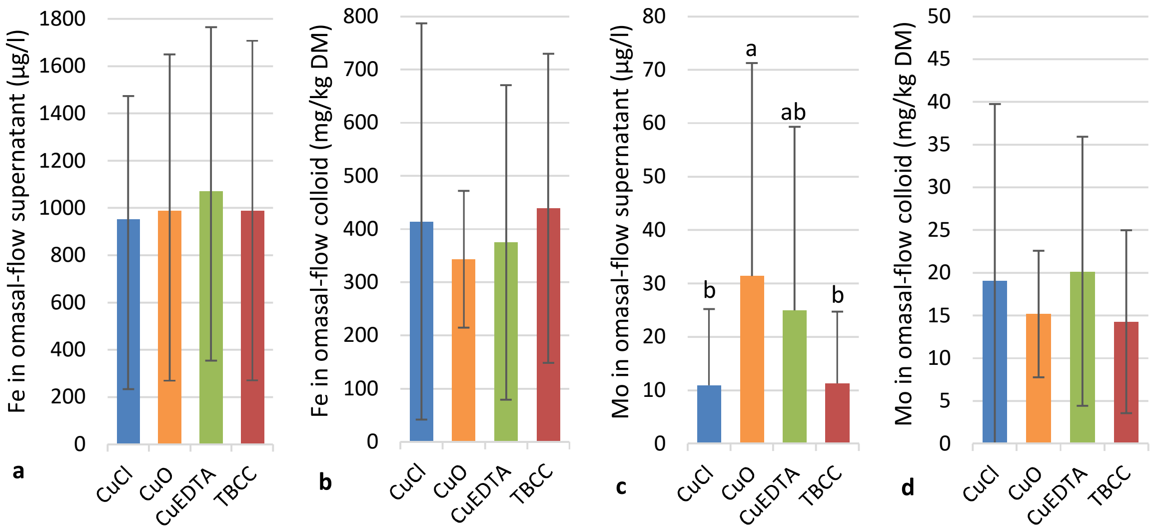

Figure 59: Iron concentration in omasal-flow supernatant (a) and colloid (b) and molybdenum concentration in omasal-flow supernatant (c) and colloid (d) for different copper sources. Error bars denote standard error of the mean. Different letters denote significant difference between treatments.

In the omasal-flow supernatant, there was (P<0.001) more molybdenum for CuO (31±40 µg/l) than TBCC and CuCl (both 11±14 µg/l) (Figure 59c). Similar to the first trial, molybdenum did not appear to accumulate in the omasal-flow, unlike copper and iron, and presented a lower concentration in this region by comparison (P<0.01) to the rumen fluid. In the omasal colloid, molybdenum concentrations were not different between the different copper sources (x̄ 17±14 mg/kg DM combined mean) (Figure 59d). Interestingly, around 90% (x̄ 89±10%) of the molybdenum was associated with the omasal supernatant but this did not differ by copper source. Molybdenum had no significant difference between the novel and steady state days for omasal-flow supernatant, but did have (P<0.001) more molybdenum in the omasal colloidal fraction on the novel day for CuEDTA and CuO (11±6 and 10±3 mg/kg DM respectively) than for CuCl and TBCC which both contained 6 mg/kg DM (±2, data not shown).

No significant differences were found between sulphur concentrations throughout the trial. In general, sulphur was associated with the supernatant of the rumen and omasal-flow fluid.

Similar to molybdenum, no accumulation of this element occurred in the omasal-flow in comparison to the rumen fluid, in fact the concentration appeared to decline ($\bar{x}$ 58±30 vs $\bar{x}$ 15±19 mg/l respectively).

Copper concentration in the faeces did not differ significantly by copper source (Figure 60). Numerically, CuO had the highest concentration (100±143 mg/kg DM), followed by CuEDTA (78±93 mg/kg DM), TBCC (46±21 mg/kg DM) and CuCl (44±20 mg/kg DM). More ($P<0.05$) copper was lixiviated from TBCC (28±10%) and CuCl (13±2%) than CuEDTA (10±2%) and CuO (8±1%).

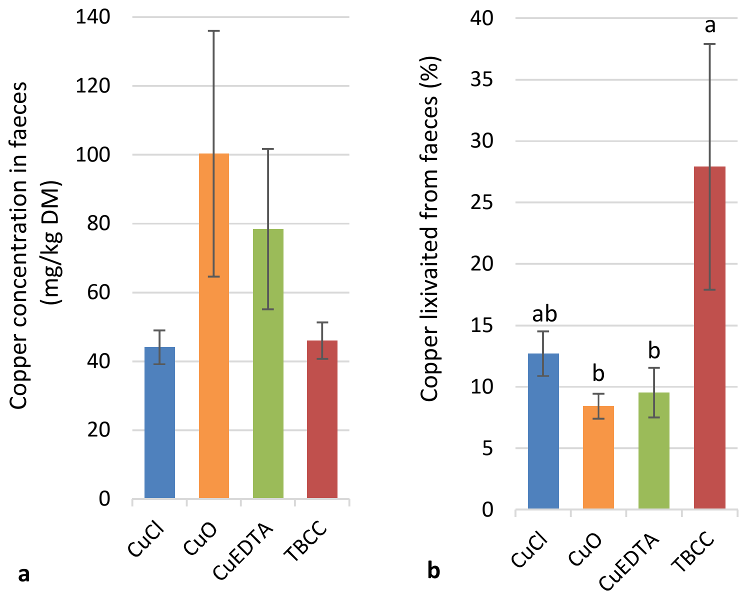

Figure 60: Copper concentration in faeces (a) and copper percentage lixiviated from faeces (b). Error bars denote standard error of the mean. Different letters indicate significant difference by treatment.

There was no difference in faecal iron concentration between the different copper sources ($\bar{x}$ 256±97 mg/kg DM combined mean) and there was also no difference in lixiviation for iron (Figure 61 a&b). Although TBCC lixiviated the most (14±4%), followed by CuCl (11±2%) and lastly CuO and CuEDTA (both 9±1%) and in both parameters there was an ($P<0.0001$) effect of period.

Faecal concentration of molybdenum did not have any significant difference between the different copper sources (x̄ 14±4 mg/kg DM combined mean) (Figure 61c). Significant differences were observed between the periods (P<0.01). Although not significant, TBCC and CuCl lixiviated the most molybdenum (both 21±4%) followed by CuO and CuEDTA (both 14±2%) (Figure 61d) and lixiviated percentage was also affected by period (P<0.0001).

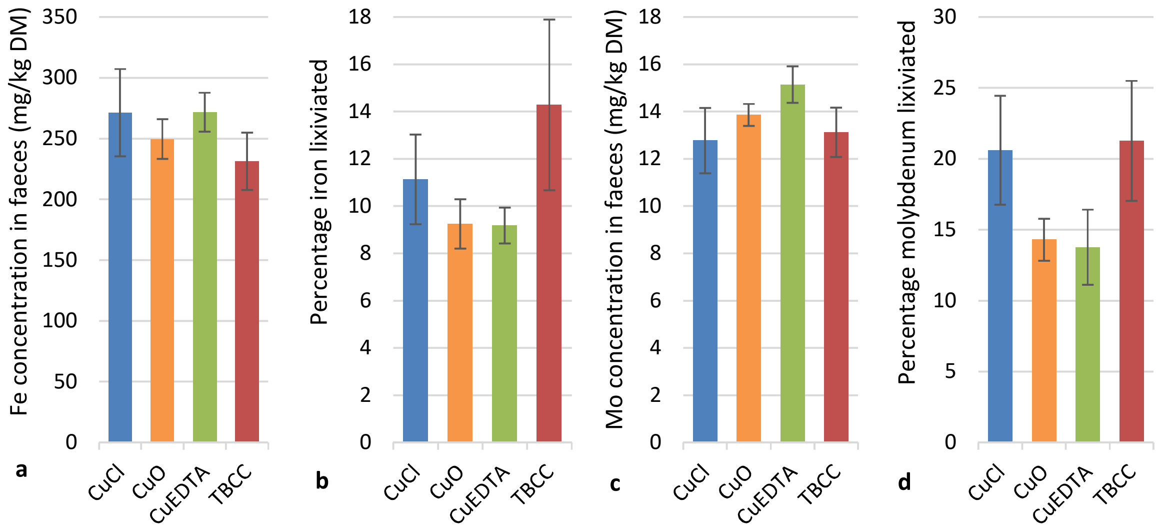

Figure 61: Iron concentration in faeces (a) and percentage lixiviated (b). Molybdenum concentration in faeces (c) and percentage lixiviated (d). Error bars denote standard error of the mean. No significant differences reported in this data.

8.4.2 Systemic parameters

Plasma copper had no significant difference between treatments. Throughout the trial, the plasma copper status remained within normal range. The range in plasma copper was 11-12 µmol/l (x̄ 12±2 µmol/l combined mean) across the treatments with CuEDTA at the top end of the scale. There were no significant differences in TCA insoluble copper concentration, serum caeruloplasmin activity or Cp:Cu ratio throughout the trial. TCA insoluble copper concentration ranged from -2 to -3 (x̄ -2.3±1.9 µmol/l combined mean); which was not suggestive of thiomolybdate formation and very few individual data points were <1. Caeruloplasmin activity was maintained within the range 17-22 mg/dl (x̄ 20±9 mg/dl combined mean) representing the lower end of normal range. The ratio of Cp:Cu was maintained between low to marginal-low status (range 1.5-1.8, x̄ 1.7±0.8 combined mean). Plasma molybdenum (Figure 62Figure) was (P<0.01) higher for CuO (0.9±0.2 µmol/l) than all the other sources (x̄ 0.7±0.2 µmol/l combined mean) and was significantly different between periods (P<0.05). SOD was also not significantly different between the copper sources. SOD concentrations were within the range 1,832-1,879 U/g Hb (x̄ 1,849±198 combined mean) with CuO at the lowest point of the scale and TBCC the highest (1,832±224 and 1,879±236 U/g Hb respectively). These results were all within the deficient range (<2,000 U/g Hb) and lower than the previous *in vivo* trial (data not shown).

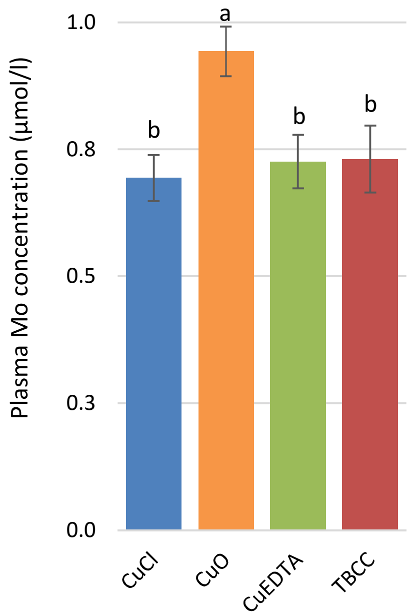

Figure 62: Variation by copper source for plasma molybdenum. Error bars denote standard error of the mean. Significant effects of treatment denoted by different letters.

8.5 Discussion

8.5.1 Behaviour of differing copper sources through digestion

Throughout the trial copper oxide (CuO) behaved as expected for an insoluble, largely unavailable copper source (Kegley and Spears, 1994). This copper source was included as a negative control for comparison to the other copper sources. It was anticipated that copper oxide would retain a low solubility in the rumen and omasal-flow supernatant and colloidal fractions; with the majority remaining as precipitate with the solid phase (Langlands *et al.*, 1989), and its absence from the rumen supernatant and colloid linked to its precipitation into the solid phase resulting in its loss from the rumen fluid sample. This was corroborated in the data gathered from this trial.

Similar to the earlier trial, the majority of copper (~80%) appeared within the colloidal fraction. This pattern appeared consistent across each of the copper sources and is consistent with the theory for both labile and thiomolybdate-bound copper to remain with the colloid (Price and Chesters, 1985; Price *et al.*, 1987).

It was expected that as a result of this low solubility and availability through digestion that there would be a relatively high faecal concentration of copper from copper oxide and that little of this copper would be solubilised through lixiviation with water. Indicating that throughout digestion the copper oxide had remained both insoluble and essentially unavailable, and had subsequently been egested. All of these effects were confirmed in the present work. Copper oxide also had the highest ($P<0.001$) plasma molybdenum concentration, suggesting that as an insoluble, largely unavailable source it was unable to inhibit molybdenum absorption into the bloodstream to a greater extent than the other sources.

Copper chloride (CuCl) was included as an example of a soluble, ionic copper source. This copper source has been demonstrated to be highly soluble and therefore potentially highly labile and interactive in the rumen (Ivan *et al.*, 1990; Ledoux *et al.*, 1995; Ward *et al.*, 1996). It was anticipated that in the rumen this source would initially be soluble or available within the colloidal fraction; possibly declining into the unmeasured solid phase as the copper interacted with antagonists. The copper chloride was found to be significantly more soluble than the copper oxide in the rumen supernatant and was also present in a higher concentration in the rumen colloid; which could potentially be labile copper or copper bound to thiomolybdate. The disappearance pattern of copper from the rumen could not be distinguished between normal passage rate and interactions causing copper to move into the non-colloidal solid phase.

In the omasal-flow, it was expected that the copper chloride, as a simple, soluble ionic source, would have fully interacted giving lower concentrations in both the supernatant and colloidal fraction in this region. This appeared to be true as the concentration did not differ significantly to copper oxide and was numerically lower in both cases. In the faeces, it was expected as a result of interaction, copper chloride would have a high faecal copper concentration alongside a low lixiviation percentage. While lixiviation was low, so was faecal copper concentration, although faecal copper concentration was not significantly different between the copper sources. This suggests that copper chloride is highly sensitive to interactions during digestion; as expected based on its solubility established in the previous work in Chapters 3 and 4, and as expected from the literature discussed in Sections 3.1.2 and 3.1.3. However, the faecal copper concentration suggests more copper than expected from simple ionic sources may be absorbed where antagonists are present.

Copper EDTA (CuEDTA) was introduced into the experimental design to establish if the effects of chelation would give this source protection from interactions in comparison to soluble ionic sources (Suttle and Price, 1976; Ammerman *et al.*, 1995; Ding *et al.*, 2011). It was hypothesised from the information in Section 3.1.3, that this source would retain lability and solubility greater than copper chloride and would yield a lower faecal concentration alongside a higher lixiviated percentage. However, in this study copper EDTA was not significantly different to copper oxide in the rumen supernatant; yielding the second lowest soluble value. This pattern was also true in the rumen colloidal fraction, although, this was not significantly different to all the other copper sources. In the omasal-flow supernatant its concentration was low; and not significantly different to both copper oxide and copper chloride. In the omasal colloid, copper EDTA had the lowest concentration of all the copper sources tested; although this did not differ significantly from copper oxide and copper chloride. The faecal concentration of copper EDTA was also high, second highest after copper oxide and had the second lowest lixiviated percentage, after copper oxide. These results suggest that the chelated properties of copper EDTA provided no protection from interactions during digestion and that it performed no differently to copper oxide throughout the trial. These findings support the work of

Quagraine, (2002) (discussed in Section 6.1.5), who found that *in vitro* copper EDTA only had limited ability to compete with thiomolybdate, ultimately displacing the copper from the EDTA.

Tribasic copper chloride (TBCC) was tested to determine if hydroxy minerals gave any of the protection to interactions that have been hypothesised (discussed in Section 3.1.3). Work in Chapters 3-4 demonstrated that this copper source had a low solubility under *in vitro* conditions and it was theorised that this mineral would exhibit a low solubility and lability in the rumen fluid, due to its low solubility at near-neutral pH (Arthington, 2015). The hypothesis continued that TBCC solubility may increase a little in the omasum and become more soluble in the acidic conditions of the abomasum; allowing it to be available for absorption in the small intestine (Arthington, 2015). Evidence of this mode of action would result in a reduced faecal copper concentration which could be highly lixiviated, while this did not appear to be entirely the case for this mineral, its behaviour *in vivo* was interesting. In the rumen fluid supernatant it gave the highest solubility; significantly higher than copper EDTA and copper oxide; behaving similarly to the soluble source, copper chloride. In the rumen colloid too, TBCC had the highest concentration (significantly more than copper oxide). This suggests that TBCC may become more labile and more soluble under rumen conditions than anticipated, despite the rumen maintaining a more pH neutral environment than the abomasal-simulated conditions which failed to release soluble copper from TBCC in Chapter 4, although further work is required to test this theory. The average solubility for TBCC of 111 µg/l (±82) reported here is similar to data from a trial of comparable design by Caldera *et al*., (2019) who reported a solubility for tribasic copper chloride in rumen fluid supernatant of ~180 µg/l without an antagonist challenge. Interestingly, copper sulphate in rumen fluid supernatant without antagonist challenge reported ~580 µg/l (Caldera *et al.*, 2019), which was far in excess of the 106 µg/l (±103) reported for copper chloride in the present work. This difference in data highlights the impact of the presence of antagonists on copper solubility, especially for soluble ionic sources, as the dietary copper inclusion in Caldera *et al*., (2019) was also lower than the present work (10 mg Cu/kg DM vs 16 mg Cu/kg DM).

The work of Caldera *et al.*, (2019) also found that steers had a significantly higher concentration of copper in the solid phase at time points sampled within 4 h post rumen dosing from copper sulphate than tribasic copper chloride. At time points beyond 6 h post rumen dosing TBCC had more copper associated with the solid phase than copper sulphate (Caldera *et al.*, 2019). In the present trial, no significant differences were found between copper chloride and TBCC for this parameter, but similar to their findings, at 1 h post-dosing CuCl measured numerically higher in the solid phase and TBCC measured numerically higher at the subsequent time points (3, 5 & 7 h) post-dosing. This difference in strength of effect may be attributed to the difference in collection method for rumen contents. In the present trial, rumen fluid was collected from an undisturbed rumen using the *in situ* tubing described in Section 5.3.2.2 and separated into supernatant and colloidal fractions. In the trial of Caldera *et al.*, (2019) a grab sample of hand-mixed rumen contents was collected giving a greater inclusion of solid phase particulate, but representing a more variable sample due to the disturbance of rumen contents and difference in sample collection position.

In the same trial, the distribution and percentage of soluble copper in the supernatant was significantly higher for the copper sulphate treatment than the TBCC treatment (Caldera *et al.*, 2019), and supported the similar findings of Genther and Hansen, (2015) who also reported a higher solubility of copper sulphate in comparison to TBCC in rumen fluid. The present work did not find any significant difference between ionic copper chloride and TBCC in rumen supernatant distribution, or percentage soluble fraction. It is possible that copper sulphate may behave differently in the rumen to copper chloride which may explain their findings. However, it is also possible that in the presence of an antagonist containing diet these differences are diminished.

The pattern of lability and solubility of tribasic copper chloride also continued through into the omasum. The omasal-flow supernatant had the highest concentration; which was significantly greater than copper EDTA and copper chloride. In the omasal colloid TBCC also had the highest copper concentration; which was significantly more than all the other copper sources, suggesting that the solubility and lability of this copper source may increase further as digestion progresses. However, Caldera *et al.*, (2019) found that there was no difference between copper sulphate and TBCC in the soluble concentration of copper in duodenal digesta and that this parameter was highly variable. It is unclear if the difference in ionic source (sulphate vs chloride) or absorption could account for this lack of difference or if this effect would be demonstrated in the present trial if duodenal digesta had been collected.

Supporting the hypothesis, faecal copper concentration from TBCC was low and its lixiviation high. Thus indicating that this copper source is able to avoid interacting with an antagonist challenge during digestion, but may become available much earlier in digestion than anticipated. Leading to the question of how it avoids interactions; which heavily affected copper EDTA and copper chloride which were expected to be soluble if antagonists were not present, in both the rumen fluid and omasal-flow. Future work to study this is suggested in Section 9.7.1.

In each of the sample periods, sample day two gave higher elemental concentration for the rumen and omasal fluid; consistent with steady state in comparison to novel introduction. Elemental concentrations also tended to be lower in the first trial period, although this was not consistently reported as a significant factor. This may have been due to the shorter acclimation time onto the basal diet and steady state having not quite been reached at the end of this trial period.

8.5.2 Effects of copper source on antagonists

Copper concentration from copper EDTA was lower than expected for a highly soluble copper source in the rumen fluid supernatant, however, iron was significantly higher in the rumen fluid supernatant where copper EDTA was used in contrast to the other copper sources. The effect observed in Chapter 5 where iron appeared able to pull copper into the supernatant was not directly observable in this trial; as all treatments had the same iron concentration. Yet, the increased iron in the rumen fluid supernatant where copper EDTA was used, may indicate that copper was sequestered from the EDTA by thiomolybdate, according to the hypothesis of Quagraine, (2002) (discussed in Section 6.1.5), leaving the EDTA unbound, which subsequently exerted its high affinity for iron, as Fe^{3+}, forming a soluble iron-EDTA compound (Xue *et al.*, 1995). However, this does not account for the significantly lower percentage of iron in the soluble fraction where copper EDTA was used, suggesting rather than displacing the copper from the EDTA, the iron may have interacted with this source forming both an insoluble complex and a soluble one.

In the omasum, there were no significant differences between the copper sources in terms of iron distribution. Although, the pattern for a higher concentration of iron in the supernatant where copper EDTA was used was still present in comparison to the other copper sources. The percentage of soluble iron for copper EDTA now accounted for the largest soluble fraction; although this was only significantly different to copper chloride. There were no significant differences in the iron content of faeces. But, in support of the ability of iron to create both a soluble and insoluble interaction (previously hypothesised in Section 5.5.1), the faecal iron concentration was the highest from copper EDTA and lixiviation the least. Suggesting that despite a higher concentration of iron present in the faeces less iron was potentially available.

There were no significant differences for molybdenum distribution in the rumen supernatant or colloidal fractions. Numerically, copper oxide had the highest molybdenum content in the supernatant and the highest in the colloid; suggesting that there may be little insoluble copper-thiomolybdate formation, and subsequent precipitation into the solid phase, potentially allowing thiomolybdate to form and become absorbed systemically with minimal detoxification by copper oxide. It is also possible that molybdate was absorbed as the current analytical methods are unable to distinguish the difference between molybdate and thiomolybdate (discussed in Section 9.7.3). A significantly higher proportion of molybdenum was found in the rumen supernatant for copper oxide in comparison to copper EDTA; which may also support this theory.

There was no significant difference in the distribution of molybdenum in the faeces, but copper EDTA had the highest faecal molybdenum concentration and had the lowest lixiviated percentage. Which may support the conclusion that this compound was highly subject to interactions from both iron and molybdenum-sulphur.

There were no significant effects on sulphur in this work. However, there was one notable change which was present but less obvious in the current work in comparison to the work conducted in Chapter 5; the disappearance of sulphur from the omasal-flow fluid in comparison to the rumen. The observed decline of both molybdenum and sulphur between the rumen and the omasal-flow fluid could potentially be linked to the formation and absorption of thiomolybdate. Although, this theory does not account for the accumulation of potentially labile copper in this region.

8.5.3 Effects of copper source on systemic parameters

The blood parameters in this trial appeared to be subject to similar problems as those discussed in Section 5.5.2. In this trial, there were no treatment effects on SOD. The medium-term nature of this parameter was not expected to be affected by the introduction of antagonists over the short-term duration of the trial. Interestingly, SOD values throughout this trial were lower than those reported in Chapter 5; which remained within normal range. The values in the present work were all notably below 2,000 U/g Hb, the threshold for deficient status (Telfer *et al.*, 2004). While these values are able to show a decline between the two trials, they may not be accurate representations of SOD activity thresholds due to the influence of the animal’s age on this parameter (Giergiel and Kankofer, 2014).

Copper chloride had normal values for caeruloplasmin activity, alongside plasma copper status in normal range. Although lacking in statistical difference between the treatments, this was the only copper source to report a Cp:Cu ratio on the diagnostic threshold for systemic thiomolybdate presence; which is in agreement with the digestive data and conclusions. Similar to the first *in vivo* trial, TCA insoluble copper concentration did not provide evidence to support thiomolybdate formation for any of the copper sources used, despite notable measures of plasma molybdenum present. It is possible that once more, an unknown process in cattle has prevented the formation of TCA-insoluble copper in the plasma (Raisbeck *et al.*, 2006), which is supported by the data gathered from the NUVetNA analysis service.

Copper oxide also had caeruloplasmin activity within normal range, alongside a normal plasma copper concentration, and most interestingly, a normal Cp:Cu ratio which did not suggest thiomolybdate formation for this copper source. However, the most significant effect was the highest elevation of plasma molybdenum in this copper source; which would support the theory that copper oxide remains essentially unavailable and does not detoxify thiomolybdate allowing both molybdate and thiomolybdate absorption.

Copper EDTA had one of the highest caeruloplasmin activity values which was well within normal range and not as high as the threshold for an acute phase response (35 mg/dl, NUVetNA reference range). Combined with the highest plasma copper status, also within normal range, a normal Cp:Cu ratio, and negative TCA insolubility, it appears this copper source was equally as able as TBCC to prevent molybdenum absorption; in opposition to the digestive results.

Tribasic copper chloride also reported a caeruloplasmin activity within the normal range and normal plasma copper status. It was interesting to note that this copper source reported a Cp:Cu ratio of 1.6 which falls in the 'grey area' which is not considered conclusive evidence of thiomolybdate formation, but is also not considered normal (Telfer *et al.*, 2004).

These results suggest that caeruloplasmin activity, plasma copper status and TCA insoluble copper concentration were unable to detect differences between these copper sources. Potentially this is because no differences exist between these copper sources for these parameters, or alternatively, the starting copper status of the animals and short duration of the trial may have masked any potentially observable effects. For these same reasons, the other blood parameters cannot provide firm evidence, but do suggest that copper chloride was subject to thiomolybdate interaction through its reduced Cp:Cu ratio and that copper oxide was the least effective copper source at preventing thiomolybdate or molybdate absorption. All of the copper sources showed a higher plasma molybdenum than the treatments which did not contain molybdenum in the previous *in vivo* trial. Indicating that none of the sources were able to entirely eliminate absorption of molybdenum, but some may have a greater ability to prevent thiomolybdate absorption. The non-antagonist treatment in the Chapter 5 trial (data in Section 5.4.5) was highly variable (0.2±0.03 µmol/l) but gave a plasma molybdenum concentration far less than those reported across all the copper sources in this trial. The combined antagonist treatment from the trial in Chapter 5 had concentrations closest to those in the present trial, and reported a highly variable plasma molybdenum concentration of 1.6(±0.1) µmol/l, which was also notably greater than those observed in the present work (range 0.7-0.9 µmol/l). While these trials cannot be directly compared, due to differences in both copper and antagonist concentrations in the treatments and basal feed, combined with high variation, they do support the notion that none of these sources prevented molybdenum, and likely thiomolybdate, absorption into the blood stream.

8.6 Conclusion

None of the copper sources tested appeared able to entirely prevent the absorption of molybdenum into the bloodstream, although feed grade copper oxide allowed significantly more to be absorbed, the others performed similarly; suggesting they may have some ability to prevent thiomolybdate absorption. Copper EDTA did not prove to be a more stable or available copper source than copper chloride, despite its stronger chelation. Tribasic copper chloride appeared to be more soluble than expected in the rumen and omasum, despite not behaving as the theory suggests. Data in this study supports the proposal that tribasic copper chloride may be more available under antagonist challenge; as a lower faecal copper concentration was found, suggesting a higher absorption. A higher percentage of copper from tribasic copper chloride in faeces could also be solubilised using water lixiviation, suggesting a greater percentage remained in a potentially available form during digestion.

9.0 Discussion

9.1 Returning to the copper conundrum

Chapter 1 outlined the difficulties surrounding copper supply in ruminants. Further understanding of the nature of the interactions which inhibit copper bioavailability and a clear understanding of the modes of antagonism which effect systemic copper supply and utilisation are essential in order to provide an appropriate copper supply to ruminants. The present work aims to further understanding of these subjects through the investigation of several key themes. The key findings are outlined in context below followed by ideas on how to further develop understanding in these areas.

9.2 Understanding of copper-related problems

Despite there being many areas which are not yet well defined in regards to copper and its interactions. There is a wealth of knowledge dating from the 1950s to present day which contributes to understanding; especially for the molybdenum-sulphur interaction (discussed in Section 1.7). Despite this, much of the available knowledge does not appear to be commonplace in the farming industry. The work presented in Chapter 2 demonstrated that copper terminology is not well understood by the farming community. Potentially, the complexity of the subject contributes to the lack of clarity and understanding; as even the simplest causes of copper imbalance such as over and under supply were overlooked by the majority of participants. In support of this, many of the farmers identified molybdenum as a culprit in copper-related problems and 'copper lock-up' was familiar term to many. However, despite the familiarity of these terms, the ability to describe the nature of these interactions was limited. Thus, leading to the supposition that the information has not effectively been translated into the farming knowledge base. It is perhaps intrinsic to this problem that there is confusion among the professional advisors in this area too. The work of Black and Kendall, (2010), discussed in Section 2.1, demonstrates that vets, nutritionists and other farming advisors lacked consistency, and among published articles in the area, key terms are often used interchangeably where they would be more effectively used to define a specific aspect of this complex topic.

A key example of this is the confusion which surrounds thiomolybdate toxicity; a term commonly interchanged incorrectly with molybdenosis and secondary copper deficiency (Anke *et al.*, 2010; Bone, 2010; Laven, 2018), discussed in Section 1.7.1. The variation in response of ruminants to thiomolybdate presents difficulties in attempts to separate these conditions, however, it is important to our understanding to do so, in order to enable appropriate remedial action to be taken where copper imbalance occurs, although the lines in practice may be blurred between these conditions. In reality, these conditions may occur at the same time, potentially from the same root cause. Molybdenosis can occur as a result of a high intake of molybdenum; irrespective of sulphur content and thiomolybdate formation, giving rise to clinical signs linked to digestive scouring and reproductive inhibition (discussed in Section 1.7.1). The presence of sulphide in the rumen, even on naturally low sulphur diets, increases the chances that alongside molybdenosis some thiomolybdates will form. Depending on the availability of copper, secondary copper deficiency (commonly 'copper lock-up') and thiomolybdate toxicity can occur. Where there is labile copper in the rumen it will bind to thiomolybdate and pass through digestion as insoluble and unavailable copper; creating the copper 'lock-up' scenario and a perceived copper deficiency ('secondary copper deficiency'). In cases of true copper 'lock-up' the clinical signs synonymous with copper deficiency, discussed in Section 1.2.1, are not always consistently present. The degree to which these classic clinical signs appear may be linked to the amount of detoxification and absorption of thiomolybdate which takes place. Data from Chapter 8, has indicated that across various copper sources at 'sufficient' inclusion rates, molybdenum, likely as thiomolybdate, will appear to be absorbed in the blood plasma, leading to the third condition; thiomolybdate toxicity. This condition is more severe where labile copper is insufficient, but in all cases of thiomolybdate absorption the classic clinical signs of copper deficiency are likely to appear to some degree. The evidence in Sections 1.7, 5.1.1 and 5.1.2 demonstrates the capacity for thiomolybdate interruption to the function of copper containing enzymes and proteins, which, given the various roles these enzymes and proteins play throughout the body (discussed in Section 1.1), give rise to the ranging and varied effects often associated with copper deficiency but are better considered the signs of thiomolybdate toxicity.

In ruminants, the effects of a simple lack of dietary copper (primary copper deficiency) are difficult to define, but using other mammals as a model, may be characterised by a very low blood copper status and anaemia; induced by cytopaenia. In severe cases neuropathological changes, such as myelopathy may be present (Kaneko *et al.*, 1997; Pyatskowit and Prohaska, 2008; Menzir and Dessie, 2017). In primary copper deficiency, there may be an absence of clinical signs as even a very low copper intake would be sufficient to meet the low (~1 mg/kg) biological requirement for copper (discussed in Section 1.6). Whereas, in cases of thiomolybdate absorption clinical signs are present due to the interruption of biological function of all the systemic copper routes.

The inadequacy of the farmer's knowledge base in practice is combined with, and potentially due to, considerable demands on the farmer's time. The evidence gathered from the survey supports that farmers are well-intentioned in their endeavours but are not equipped with sufficient tools to make informed decisions. Many farmers collected data with regard to their animal's copper status and their forage, but these were not necessarily the most appropriate measures and were not used to inform decision making processes; thus failing to update supplementation strategy in line with herd or flock status, or supply, contributing to widespread imbalance in the national herd and flock. This detrimental cycle continues as the risk of imbalance is not accurately assessed or managed on an ongoing basis (Kendall and Bone, 2019). The use of broad-spectrum, long-lasting, easy to use supplements administered with key husbandry events also echoes this point. Farmers may seek to do the right thing for their animals, but are ultimately limited by their time or their understanding in their execution.

It was encouraging to find that most farmers who reported they had no knowledge of copper had underestimated their level of understanding. However, it was concerning to find that those who felt they had some level of copper knowledge had most likely overestimated their level of understanding. In fact, both of these findings may lead to problematic outcomes. Those who lack confidence in their knowledge are more likely to take advice and guidance;

attempting to further their understanding. This is desirable where the advice is accurate, but creates an issue where they may seek advice from peers who have overestimated their level of understanding or gain advice from advisors who do not consistently understand the issues themselves (Black and Kendall, 2010), leading to the spread of further confusion and misinformation.

It is important that farmers understand the terminology, antagonist modes of action, and even the subtle differences between the various conditions that arise, to enable them to accurately assess the risk of copper imbalance in their animals and provide appropriate supplemental strategies to ensure optimal copper status in their herd or flock.

9.3 The movement of copper through digestion and metabolism

9.3.1 The path of ingested copper

The work presented in Chapter 7 using XAS to identify copper and iron bonding and oxidation state through digestion appeared to be the first trial of its kind. Synonymous with current thinking on the dissociation of simple ionic compounds in the rumen (discussed in Chapter 3), the work demonstrated that none of the original ionic compound for iron or copper remained intact in the rumen. The complete speciation in the rumen was accompanied by a unique finding; the partial reduction of Fe^{3+} into Fe^{2+} and the complete reduction of Cu^{2+} into both Cu^{+} and surprisingly some Cu^{0}. As discussed throughout Sections 1.1, 6.1.5, 6.5 and 7.5 the oxidation state of copper has a great influence on its chemical properties and interactions. The finding of elemental copper has not previously been reported thus, the mode of its formation is largely conjecture (Section 7.5). Despite this, its role in the dynamics of ruminant digestion as well as its impact on antagonist pathways and normal absorption routes requires further study (discussed in Section 9.7.1).

The work conducted throughout Chapters 3-8 demonstrates the importance of studying the behaviour of copper sources *in vivo* rather than *in vitro,* and the use of XAS on samples provides a greater insight into the chemistry of the interactions which take place than other, more routine, analysis methods. The high reactivity of Cu^{+} combined with the similarity of XAS spectra to thiol based compounds suggests that this may be one of the most biologically relevant competing ligands in the rumen; consistent with each of the known major antagonist pathways and the work conducted in Chapters 3 and 4 which demonstrated a clear affinity between sulphide ligands and copper.

A further novel finding arising from the antagonist feed work in Chapters 5 and 8 was the increased concentration of copper found in the omasal-flow in both *in vivo* trials. The accumulation of around 50-times more copper than was present in the rumen fluid poses new implications for the modes of interaction and antagonism. It was interesting to note that the accumulation of copper was accompanied by a lesser accumulation of iron (~2x) and a decline in molybdenum (~0.5x) and sulphur (~0.25x) concentration from the rumen. A possible hypothesis for the accumulation of copper may be due to the presence of elemental copper (Cu^0). As an insoluble and unreactive component of the digestive matrix it may associate with the digestive 'sludge'; the thicker, insoluble, fine particulate, of the colloidal fraction in the liquid. The elemental copper would be steadily washed into the omasum through rumen contractions but collect here due to its insolubility, before progressing at a slower rate through the remainder of the digestive transit. It is also possible that this same process occurs for an insoluble iron-copper compound; explaining the increase in iron as well as copper found in this region.

The work using XAS on sheep digesta presented in Chapter 7 also demonstrated for the first time that changes are able to take place to the copper-containing complex as it moves further through digestion; having changed notably in its local co-ordination bonding between the rumen and jejunum. Although, the complexes remain un-identified, they are likely to be more complex than previously thought and may also contain chloride and oxygen in addition to copper, iron and sulphur in a structure not too dissimilar from bornite.

9.3.2 Adaptation to copper supply

The importance of maintaining a copper balance and its essentiality for mammalian life was highlighted in Section 1.1. However in the case of ruminants, the mechanisms in place for copper regulation do not appear suitably able to cope with oversupply leaving them susceptible to accumulation. Copper tolerance varies with species, breed and individual, and has been shown to be a heritable trait (Cutler and Jones, 2003; Simpson *et al.*, 2006). This suggests that the patterns of copper tolerance may have a basis in the animal's evolutionary history. Historically, due to the prevalence of interactions which limit copper absorption and availability, ruminants may have evolved not to require the mechanisms to deal with oversupply. Instead, expressing the capability to evolve to restricted copper environments. Examples of this are found in both sheep and cattle from copper deprived areas:

North Ronaldsay sheep are found only on the shoreline of the Orkney Islands. Their diet is unique, consisting of seaweed predominantly from the brown kelp families (*Laminaria*) (Hansen *et al.*, 2003). This forage is known to be very low in copper (<5 mg/kg DM), but also very low in molybdenum (<0.5 mg/kg DM) and iron (~28 mg/kg DM) (Haywood and Britt, 2008). Despite this, copper deficiency is not reported in this breed. In fact, they are noted to have an increased efficiency for dietary copper absorption; up to a 10-fold increase, in comparison to mainland counterparts (Haywood and Britt, 2008). Their increased ability to absorb copper is attributed to the over-expression of the copper transporters, which allow for the facilitated uptake of copper from the intestine (Haywood and Britt, 2008). However, this effective uptake has led to them becoming extremely copper sensitive. This breed is so sensitive to copper that even typical herbage, relatively high in molybdenum, can cause haemolytic crisis alongside liver copper in excess of 20,500 µmol/kg DM, while their counterparts, (Cheviot x North Ronaldsay) maintained normal serum copper concentrations without clinical signs (McLachlan and Johnston, 1982).

Zebu (*Bos taurus inidicus*) are a sub-species of cattle originating from Southeast Asia and are kept across many parts of Africa where copper deficiency is perceived to be common (Dermauw *et al.*, 2013, 2014). The East African Rift Valley stretches across most of East Africa from Djibouti, through Ethiopia, Kenya, Rwanda, Tanzania and Zambia, ending in Mozambique (Faye *et al.*, 1991). This immense stretch of land is known for its high sulphur (>2 g/kg DM) and molybdenum (>2 mg/kg DM) forage content (Faye *et al.*, 1991; De Cuyper, 2013; Dermauw *et al.*, 2013), as well as a low copper content (~6 mg/kg DM). Zebu grazing under these conditions appear more resilient to copper deficiency than Zebu x Holstein Friesian counterparts; maintaining higher plasma copper and liver status (Dermauw *et al.*, 2014). The Zebu, exhibit a greater expression of copper importers and chaperones in the enterocyte secretory pathway, alongside a higher hepatic expression of the main chaperones for cuproenzyme and protein synthesis; including almost twice as much metallothionein (De Cuyper, 2013; Fry *et al.*, 2013; Dermauw *et al.*, 2014). Unlike the North Ronaldsay, Zebu are not noted for their copper sensitivity and the recommended MPL for them is no different to their *Bos taurus* counterparts (de Campos *et al.*, 2016). The difference in sensitivity to elevated copper has not been investigated. However, there are two main differences between these animals. Firstly, the North Ronaldsay is subject to primary copper deficiency, whereas the Zebu in addition to low copper content, have the presence of antagonists in their forage. Secondly, the Zebu appear to produce more metallothionein than their counterparts, whereas the North Ronaldsay appeared to produce a little less metallothionein (not significant) than Cambridge sheep in response to increasing copper concentrations (Simpson *et al.*, 2006).

It possible that over time ruminants have previously evolved to cope which chronic copper undersupply, but it is perhaps only through our intervention in providing available copper as part of farming practice that introduces them to copper accumulation; a condition which they did not historically encounter and are ill-equipped to deal with.

9.3.3 Copper handling in the rumen

The rumen microbes are predominantly anaerobic and highly oxygen sensitive (discussed Section 1.5). It is thought that many of the ruminal bacterial species evolutionary diverged before the presence of dioxygen (O_2); which not only limits their ability to cope with oxygen, but also makes them highly copper sensitive (Ridge *et al.*, 2008; Pontel and Soncini, 2009; Solioz *et al.*, 2010; Richter, 2011). The evolutionary requirement for copper in living organisms was influenced by the increase of atmospheric dioxygen on the Earth ~2 billion years ago (Solioz, 2002; Boal and Rosenzweig, 2009). Prior to this, it is thought that iron played the redox role in many of the reactions that involve copper today (Argüello *et al.*, 2013). As dioxygen persisted, iron became stable in its oxidised form (Fe^{3+}) and was no longer functional, allowing soluble Cu^{2+} to assume its role (Rubino and Franz, 2012). Thus combining the presence of an oxygenated atmosphere and the development of cellular copper handling mechanisms; which are now ubiquitous across eukaryotic life (Zhou and Gitschier, 1997; Lee *et al.*, 2000).

Many prokaryotes are able to utilise copper; possessing copper transporters and copper-containing proteins (Ridge *et al.*, 2008). Bacterial homologues exist for several of the copper transporters recognised in eukaryotic cells (Solioz and Odermatt, 1995; Rensing and Grass, 2003). These oxidases and copper chaperones are common in aerobic bacteria, but are lacking in anaerobes; as they are only considered to be useful in the presence of oxygen (Solomon *et al.*, 2008).

In order to enter all cells, copper is required to be in Cu^{+} form to cross cell membranes into the intracellular environment (Solioz and Stoyanov, 2003). Membrane reductases in 'copper-using' species of eukaryotes and prokaryotes exist to ensure uptake can take place (Rensing and Grass, 2003; Sinani *et al.*, 2007; Collins *et al.*, 2010). However, in the rumen Cu^{+} is already the predominant form; allowing it to freely cross the membrane into bacterial cells without active import (Clark, 2008). *In vitro* studies have demonstrated that copper is particularly toxic to rumen micro-organisms (McNaught *et al.*, 1950; Hubbert, 1957). However, it remains undefined at what concentration the copper toxicity threshold is reached, as the population is widely varied (discussed in Section 1.5) and some species of rumen bacteria express rudimentary copper efflux mechanisms to combat its presence (Rensing and Grass, 2003; Ridge *et al.*, 2008; Subashchandrabose and Mobley, 2015).

Therefore, it is likely that the binding of copper to antagonists in the rumen is beneficial to the rumen's bacterial population. Furthermore, the most abundant sulphur-reducing bacteria present in the rumen belong to the genus *Desulfovibrio* which use the dissimilatory pathway of sulphide production (Peck, 1961). The dissimilatory pathway is considered among the earliest energy-yielding processes to sustain life and is common among archaea; single-celled micro-organisms with a structure similar to bacteria, that populate extreme environments, such as the ruminant digestive tract, and obligately anaerobic bacteria; notably those which evolved before the presence of atmospheric oxygen (Grein *et al.*, 2013). The presence of these bacteria, with their inability to tolerate copper and ability produce a substance which would protect them from its deleterious effects raises the question: Do the sulphur reducing bacteria promote copper binding in the rumen as a protective mechanism? If this is the case, the copper conundrum may never reach a satisfactory conclusion. Previous work using sulphur-reducing bacterial species (*Desulfovibrio* spp.) has documented their ability to 'safeguard' environmental copper (Sani *et al.*, 2001; Sheng *et al.*, 2011; Ayangbenro *et al.*, 2018), but has not demonstrated if they are able to increase sulphide production in response to rising copper concentration.

9.3.4 Copper in the liver

Section 6.1.2 discussed the further complexities of liver accumulation in ruminants. The difficulty in determining the level at which copper accumulation translates into the risk of haemolytic crisis appears individually unique and worthy of further research. The work presented in Chapter 6 on liver copper compounds, highlighted the preference for copper from sulphur containing ligands. The likely storage compound for copper in the high copper status ruminant liver is metallothionein (discussed in Section 6.1.1). This copper buffer is slow to respond and appears to plateau in ruminants, but does still appear to play a key role in copper binding in both bovine and ovine livers which exceed 8,000 µmol/kg DM; which indicates an elevated hepatic copper status potentially within the threshold for haemolytic crisis. The similarity of spectra for metallothionein was more pronounced in the bovine liver, which did appear to differ to the ovine liver in its average composition. In line with previous work (discussed in Section 6.1.4), this result suggests that cattle are able to use their metallothionein capacity more effectively than sheep; which suggests that in elevated liver copper status there may be a role for glutathione to buffer copper which exceeds the carrying capacity of metallothionein, for sheep more so than cattle. The data from Chapter 6 showed some similarity to glutathione in both species, although this appeared more relevant to sheep. It has been previously suggested that glutathione may act as a low-molecular ligand to stabilise Cu^{+} in the intracellular environment (Palumaa, 2013). Initially its role was considered to chaperone copper upon entry to the cell (Freedman *et al.*, 1989), but more recent cellular toxicity work suggests that its buffering capacity to deal with excess beyond metallothionein capacity is biologically relevant (Saporito-Magriñá *et al.*, 2018). This potentially explains the ability of copper to accumulate in some livers to concentrations well in excess of 8,000 µmol/kg DM in apparently healthy animals. Interestingly, the data from Chapter 6 also created good fits including molybdenum in sheep and shared spectral similarity to copper-thiomolybdate from thiomolybdate treated LEC rat livers in both species. The work presented in Chapter 6 failed to produce a suitable copper-thiomolybdate for comparison. Subsequently, the data was compared to spectra from thiomolybdate treated LEC rat livers (Zhang *et al.*, 2009) to try and establish if this compound was likely to be present. However, LEC rat livers may not be the best model to use for comparison as they are a mutant strain, missing ATP7B; an important copper chaperone in the secretory pathway, responsible for directing copper to be incorporated into caeruloplasmin (Kaneko *et al.*, 1997; Failla, 1999; La Fontaine and Mercer, 2007; Suttle, 2012). While the secretory pathway is diminished in ruminants, especially sheep, ATP7B is still functional. In fact, unlike any other species, sheep express two types of ATP7B (Lockhart *et al.*, 2000). One, the homologue of rodent and human ATP7B; the other, comprising only ~10% of total metallothionein was a unique version (Lockhart and Mercer, 2001). It was initially proposed that the novel sequence in the unique ATP7B (termed sATP7B) may act as a targeting motif directing copper to the lysosomal membrane (Lockhart and Mercer, 2001). Allowing copper to be transported into the lysosome for storage instead of efflux as intracellular copper concentration increases (Lockhart *et al.*, 2000; Lockhart and Mercer, 2001). However, functionality tests suggest that both ATP7B variants are able to function normally in copper regulation with no significant difference in functionality between the two types (Lockhart and Mercer, 2001; Lockhart *et al.*, 2002), suggesting that this may not be the cause of the susceptibility of sheep to accumulate hepatic copper.

The similarity in sulphur ligands of glutathione, thiomolybdate and metallothionein contribute to the difficulty in determining the exact compounds present, but despite the concentration of molybdenum in the liver not exceeding normal range in the present work, the presence of copper-thiomolybdate could not be ruled out for the ovine liver.

9.4 The iron-sulphur antagonist pathway

It is widely acknowledged that iron impacts copper availability, while little is known about the mode of action (discussed in Section 1.8). The *in vitro* solubility work in Chapters 3 and 4 showed no indication of a reduction of copper solubility with iron and sulphide in comparison to the influence of sulphide alone, leading to the conclusion that the interaction is not a result of simple chemistry. The *in vivo* work in Chapter 5 exerted an unexpected effect; the movement of copper into the soluble fraction in the presence of iron. If the initial stages of the iron antagonism result in a soluble compound in the rumen, it could possibly explain why the solubility trials were unable to detect its formation. In order for this compound to pose a problem for copper bioavailability the complex would have to be too large for absorption despite remaining soluble (discussed in Section 5.5.1). This could occur if the molecular size of the soluble compound exceeded 1,000 Da or possessed other characteristics incompatible with absorption (as discussed in Section 3.5). Since these are only early indications, lacking in evidence they require further study, as suggested in Section 9.7.1 to fully establish the existence of this complex.

The current theories surrounding this antagonism (Section 1.8) both rely on the formation of an insoluble compound. Some possible evidence of this was also found in the *in vivo* work in Chapter 5, where the lesser percentage of iron, and copper in the high iron treatment, in the omasal-flow supernatant compared to the rumen implied, rather than directly evidenced, that a compound formed between them, which subsequently precipitated out of the sample. It is possible that the antagonism proceeds as a combination of these two mechanisms; potentially linked to competition from the other ligands present and potentially changing as digestion progresses.

The XAS of digesta in Chapter 7 found no evidence for iron in a copper containing compound in the rumen. Suggesting the copper was most likely bound to sulphur and other copper atoms. However, in the jejunum two pathways which included iron were successfully fitted, giving the first indication of compound formation. It was also interesting to note that this compound did not appear to consist of purely copper, iron and sulphur, it also included; oxygen and chlorine (most likely as chloride). This work indicates that the structure of the iron-copper-sulphur compound may be more complex than previously thought.

9.5 The dynamic rumen and antagonist pathways

In agreement with previous research (discussed in Section 1.7) the work conducted in Chapter 5 found molybdenum to be the most potent inhibitor of copper. The limitations of the total elemental recovery prevent thiomolybdate determination from molybdate, and the lack of correlation with blood parameters prevented the systemic markers from providing evidence of their formation.

The data gathered throughout the present work supports the theory that any soluble Cu^{2+} which enters the rumen from feedstuffs associates primarily with the colloidal fraction, where it is either immediately reduced by the rumen environment into Cu^{+} or immediately sequestered into thiomolybdate; reducing it into Cu^{+} and forming a stable, insoluble copper-thiomolybdate complex. It appears that this pathway will act at the highest affinity. Progressing in a linear trajectory from around an hour after feeding if previous formation hypothesis are correct (Quagraine, 2002). The 'free' Cu^{+} will then interact in a combination of three potential routes, shown in Figure 63. Cu^{+} is able to bind with sulphide forming insoluble copper sulphide, but at a much lower affinity than thiomolybdate; potentially only posing a problem where thiomolybdate is not present in the rumen. The copper sulphide is highly insoluble and likely precipitates out of the rumen fluid into the solid phase. Or, the Cu^{+} could be pulled into the liquid fraction creating a soluble compound with iron. Lastly, Cu^{+} could be reduced into Cu^{0}, which is slow to react and relatively inert, posing only a minimal threat to rumen microbiota through direct surface contact in comparison to Cu^{+}, and which also precipitates into the solid phase.

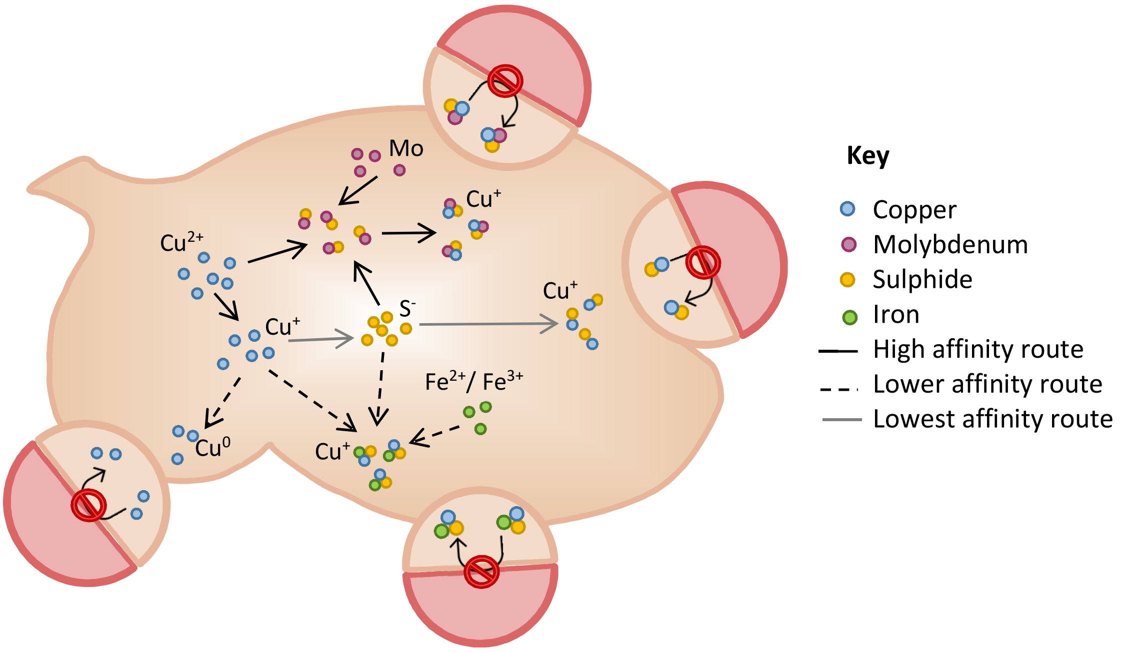

Figure 63: Potential routes of copper in the antagonist containing rumen given as an overview.

These three routes of lower affinity will likely progress in a hierarchy depleting copper from the labile pool. Due to the clearance rate and movement of the rumen an equilibrium may never be established, thus each of these pathways may continue to compete alongside the dynamics of the environment.

9.6 The influence of copper source

The simple solubility work conducted in Chapters 3 and 4 demonstrated that the effects of sulphide were purely chemical and appeared to affect each of the copper sources tested, although, this was less pronounced where initial solubility was already low. The copper sources demonstrated some similarity in the *in vitro* work between their solubility and the bioavailability studies of other workers (discussed in Section 4.5), with copper sources; sulphate, chloride, acetate and proteinate appearing both soluble and bioavailable. This suggests that the chemical properties of the copper sources discussed in Section 3.1.3 and demonstrated in Chapter 3 and 4 are an important factor in their consideration for use as a supplement. However, there were differences preventing rumen replicated *in vitro* testing from being used as a substitute for animal trials. An example of this difference was found for copper carbonate and tribasic copper chloride which both appeared relatively insoluble in the present work, while their bioavailability in previous animal work indicated they were highly bioavailable. These differences likely arise from disparity in measuring solubility over the markers for bioavailability and from the influence of the dynamic rumen environment over simple *in vitro* replication. The *in vivo* work in Chapter 5, highlighted the importance of the rumen dynamics in studying the interactions of copper and demonstrated that the effect of sulphide was less relevant biologically than the presence of molybdate alongside sulphide.

In Chapter 8, the ideas from these previous chapters were combined, with interesting results. It appeared that none of the copper sources were completely able to prevent the absorption of molybdenum into the bloodstream. Potentially two reasons exist for this finding:

Firstly, the copper sources were unable to completely bind the formed thiomolybdate, allowing its absorption. This could potentially lead to the development of thiomolybdate toxicity and its subsequent interference effects on biological copper.

Secondly, it is possible, although unlikely, that thiomolybdate was not formed allowing molybdate to be absorbed; which may have a lesser effect on biological parameters but could potentially lead to molybdenosis, as opposed to thiomolybdate toxicity (as defined in Sections 1.7.1 and 9.2). The work conducted in both chapters (5 & 8) generated sulphide to molybdenum ratios well in excess of those previously quoted as necessary to elicit thiomolybdate formation (Clarke and Laurie, 1980; Bray *et al.*, 1982b), despite the blood parameters being unable to conclusively detect them. It is most likely that a combination of these effects occurred, with the formation and absorption of some thiomolybdate and a proportion of molybdate absorption.

The limitations of the analysis are a major drawback in determining the difference between these effects, as copper is not anticipated to bind directly to molybdate, but a copper source which allows thiomolybdate absorption is potentially detrimental to the animal's health if used as a supplement. Ample rumen sulphur concentrations are integral to the formation of thiomolybdate. Sulphur concentrations were largely consistent in the measured parameters throughout both *in vivo* trials; remaining similar across treatments and across trials (Chapters

5 and 8). However, there were two ($P<0.0001$) differences in concentrations between the trials. The sulphur concentration in the rumen fluid supernatant was much lower in the Chapter 5 work (5±9 mg/l) compared to the same parameter in Chapter 8 (58±30 mg/l). The opposite effect ($P<0.0001$) was observed for plasma sulphur concentration between the trials (1,071±85 vs 916±124 mg/ml). The total sulphur inclusion was higher in the Chapter 8 trial (3 vs 4 g/kg DM). However, this notable difference in concentration may suggest that some of the rumen interactions could have been sulphur-limited in the Chapter 5 work; potentially favouring the higher affinity thiomolybdate pathway over the copper sulphide or iron pathway. This supports the hypothesis that thiomolybdate formation occurred and was absorbed, especially in the earlier work. While the increased dietary inclusion in the Chapter 8 work allowed a greater competition between ligands and wider spectrum of interactions to occur in the rumen during the later work.

Copper oxide appeared to behave in conjunction with the expectation that it remains insoluble and does not constitute a bioavailable source of copper. However, the other copper sources yielded different results to those expected. Copper chloride appeared to behave as a soluble ionic compound which participated in the antagonist interactions. Despite this, the faecal copper concentration from copper chloride was lower than expected which indicates that more copper was retained in the animal than expected.

The most interesting results were found using tribasic copper chloride and copper EDTA. Copper EDTA in previous trials was not frequently fed and was administered parenterally (discussed in Section 3.1.3). Only two trials, one in sheep and the other in cattle, have used copper EDTA as an oral supplement. The first trial conducted by Macpherson and Hemingway, (1968) compared the efficacy of copper sulphate, copper glycinate and copper EDTA in the form of drenches to replete copper deficient sheep. The trial found that copper EDTA performed similarly to the other copper sources in its ability to replete both blood copper and liver copper concentrations. The second study conducted by Dunkley *et al.*, (1968) compared copper EDTA, copper sulphate, and sodium EDTA mixed into concentrate feed fed to lactating cattle for their effects on milk copper concentration. They found that both copper sulphate and copper EDTA increased milk copper concentration equally and both failed to increase blood copper concentration.

Concerns regarding the use of this source were related to the stability of its chelation (Ding *et al.*, 2011). The stability potentially posed two problems: The first is an inhibition of bioavailability if the copper is unable to be released from the EDTA by the chaperones. The second is linked to the competition with thiomolybdate. Previous competition work established that at lower concentrations and where copper was already bound to EDTA it had some resistance to thiomolybdate (Quagraine, 2002). It was hypothesised that if this copper source was used in a feed grade supplement under conditions likely to elicit thiomolybdate formation its ability to resist this interaction could allow some thiomolybdate absorption and subsequent interruption of biological copper. Subsequently, the decision to use this source as a parenteral injection was suggested.

Despite these concerns, in the present work copper EDTA did not appear to provide any protection against the antagonist challenge in the rumen. Behaving, as the two oral studies suggested in a similar manner to simple ionic sources. The competition and excess of thiomolybdate may have been sufficient to displace the copper from the EDTA giving it similar values to copper oxide through digestion and similarly high faecal concentration with low lixiviation (discussed in Section 5.3.3.8). Suggesting it had a lower bioavailability and was more susceptible to interactions than copper chloride under these circumstances. It was interesting that despite this limited digestive performance the blood parameters appeared somewhat resistant to decline. Although, the lack of significant difference between the copper sources for blood parameters prevents this from being conclusive.

Tribasic copper chloride yielded results the most different from expected of all the copper sources tested in this work. Products of this type are marketed as a rumen ‘by-pass’ products. However, potential risks of feeding rumen ‘by-pass’ copper sources alone have been raised (discussed in Section 3.5). The lack of solubility at ruminal pH theorised to allow the TBCC to avoid antagonist interactions was clearly demonstrated in the solubility work in Chapter 4. However, *in vivo* TBCC had the highest rumen solubility of all the copper sources tested and maintained this high solubility in comparison to the other 'soluble' sources through into the omasum. This effect is interesting, it suggests that TBCC is soluble under rumen conditions, counter to the current marketing of these mineral types. However, the effects were more complex. TBCC also had the lowest faecal concentration, highest lixiviation and blood parameters close to normal range without an antagonist effect, which indicate that this source was the least affected by the antagonist challenge. The mode of its resistance cannot be attributed to the rumen 'by-pass' theory, but does pose an interesting model for further study.

9.7 Future work

9.7.1 Further copper complexing through digestion

Several questions were raised through this work in regard to the speciation and complexing of copper during digestion. The present work could be further enhanced through the XAS analysis of a greater range of model compounds which could then be used in PCA and LCF analysis (discussed in Section 6.1.6 and 7.4.3.1) with the data gathered in Chapter 7. A greater range of standards comprising known compounds containing copper, iron and sulphur, including more complex structures with chloride, may help to identify the nature of the complex formation and would also help to gain a more accurate value for the percentage of elemental copper found in the rumen.

In addition, further XAS analysis of the copper and iron K-edges in the remaining digestive regions, using the samples already gathered, could assist in mapping the progress of copper and iron through digestion. The results in Chapter 7 showed that speciation of copper occurred between the rumen and jejunum. During the animal experimental work conducted in Chapter 7, samples from the reticulum, omasum, abomasum and faeces were also collected but were not analysed using XAS. Analysis of these additional regions of the digestive tract using the same experimental design would allow the local co-ordination bonding of copper and iron to be studied; providing further insight into the nature of these changes and map their progression through the digestive process.

Additionally, these same methods could be applied to digesta which have been separated into supernatant and colloidal fractions to investigate the differences in local co-ordination bonding which may occur between the fractions of the same region. The use of XAS on supernatant and colloidal fractions of various regions of the digestive tract will also be beneficial to the determination of the presence and role of elemental copper during digestion. In particular, the results in Chapter 7 suggested that the rumen was the area of Cu^0 formation and the results from Chapters 5 and 8 suggested that copper accumulates in the colloidal fraction of the omasal-flow. It was hypothesised that the Cu^0 accumulating here may move at a slower rate through the rest of digestion due to the sieving effect of the omasum, which would also account for the loss of Cu^0 in the data from the jejunum samples. The use of fractionation of the digestive material followed by XAS analysis of the copper K-edge would help to determine which fraction of rumen fluid the Cu^0 associates with and if the copper remains as Cu^0 in the colloidal fraction of the omasal fluid in line with the present hypothesis.

Further work could also include a series of short-term animal trials of a similar design to that used in Chapter 7 (Section 7.3.1). These animals, preferentially sheep due to their ease of handling and to maintain consistency between the work, would be fed a diet high in sulphur, molybdenum and copper; aiming to gut-load the animals with thiomolybdate bound copper. To create the series of trials, different copper sources would be used to create the treatment groups. Copper sulphate; as the industry inorganic ionic source would make a good reference source, in addition to copper chloride to allow comparison to be drawn with the iron-challenge work (Chapter 7). Other copper sources such as TBCC would be interesting; as the mode of action *in vivo* does not appear to be as expected from *in vitro* work, alongside an organic source; to examine any differences which may occur due to the nature of its bonding. In each of these animals XAS analysis of prepared rumen samples could help to determine how each copper source speciates and how they participate in, or avoid, interactions.

Additional work to elucidate if a soluble iron-copper compound forms could be conducted on rumen samples. Samples of rumen fluid could be obtained from animals fed a high sulphur, iron, and copper diet using slaughter-recovery where fistulates are not available. The use of fistulated animals in this work would be beneficial but not essential. The use of slaughter-recovery, such as that described in Section 7.3.1 would also yield reliable results. The advantages of using either fistulated animals or small scale slaughter-recovery over techniques such as rumen intubation, would allow more consistent sample collection from within the regions of the rumen and allow a greater cross-section of the rumen contents to be collected and separated for analysis.

Dialysis could be employed on the fluid, similar to methods conducted in recent work (Caldera *et al.*, 2019) to mimic absorption and allow the concentration of key elements to be established. Comparison could be made between the total elemental composition, as obtained through wet-acid digestion and ICP-MS, and the dialysed, potentially absorbable, fraction. Dried pellets of rumen solid phase as either colloid or rumen mat could be suspended in a measured volume of Tris-EDTA buffer and dialysed using cellulose dialysis tubing against the same volume of pure buffer solution for ~12 h with continuous mixing. The resulting dialysed samples could then be re-dried and analysed for their elemental content using wet-acid digestion and ICP-MS for comparison. Depending on the success of such work, radio-labelled copper (^{64}Cu, $T_{½}$ 12.7 h) could also be used to try and establish how copper associates between the supernatant, colloidal and solid phases. This work could use $^{64}CuCl_2$ to identify how copper fractionates upon the addition of a pulse-dose of radio-labelled copper with feeding. In a short-term trial, using small animal numbers, housed under laboratory conditions, rumen content samples would be collected at slaughter and first be strained to obtain the solid fraction and then centrifuged using the methods described in Section 5.3.3.1 to allow the solid, colloid and supernatant to be analysed and counted separately allowing for a percentage distribution of ^{64}Cu across the fractions compared to intake at a specific time point to be calculated.

The last aspect of digestion which raised further questions during this work was the *in vivo* results from Chapters 5 and 8 demonstrating copper accumulation in the omasum. Analysis using ICP-MS of the sheep omasal contents gathered as part of the work in Chapter 7 would potentially establish if this effect is present in sheep. Further *in vivo* studies could use a 4 x 4 factorial design with treatments comprising high and low iron, molybdenum and sulphur alongside controlled copper inclusion. These studies could be conducted in fistulated animals with omasal-flow samples gathered using the same technique as Section 5.3.2.2, or as slaughter recovery trials. The slaughter recovery trial has the advantage that a greater number of animals could be used per treatment group which would aim to improve the variability and standard error of the copper data in particular, after analysis using wet-acid digestion and ICP-MS; although, the slaughter recovery method makes separation of the digesta in the omasum less accurate due to potential mixing of contents during the process. Depending on the findings of this trial, there is also potential to use radio-labelled copper as a pulse-dose, like that suggested in earlier in this section, in a similar design to the work in Chapter 8 using omasal-flow sampling in fistulated animals over a greater range of sampling time points to give a greater insight into the dynamics and time-frame of this accumulation.

9.7.2 Thiomolybdate in the liver

The further study of the potential for thiomolybdate sequestering of systemic copper and its role in high copper status livers in ruminants could be conducted through an *in vivo* trial. Trial animals could be fed a high copper, sulphur, molybdenum diet in a similar design to the Chapter 7 Section 7.3.1 work. These animals would preferentially be sheep of low-deficient liver copper status. Although, in a later study, cattle of a similar liver copper status could be used to create a contrast for species difference. A longer period of time on the diet compared to the current work to sufficiently allow uptake and hepatic copper-loading is required. A second batch of animals fed the high copper diet without elevated molybdenum and sulphur could act as a comparative control. Upon slaughter the livers would be recovered and prepared using the same techniques in Chapter 6, Section 6.3.1 and analysed using XAS of the copper K-edge, which could also be compared to the livers analysed in this work. A further study may be to analyse the same samples for the molybdenum K-edge. However, this was not conducted in the present work due to practical restrictions which would also need to be considered in any future work. To analyse the molybdenum edge the concentration would need to be >500 µM to fall above the beamline detection limits. This concentration is estimated to be equivalent to feeding >150 mg/kg DM of molybdenum to sheep. The MPL has recently been advised at 2.5

mg Mo/kg complete diet in sheep, equivalent to 2.8 mg/kg DM at 88% DM (Bampidis *et al.*, 2019). Thus, to feed in excess of 150 mg/kg DM poses serious concerns to the animal's health whilst on the trial. In addition, samples which are well in excess of the detection limit are also known to give a low fluorescent yield due to the electronic configuration of the molybdenum atom. This makes the resulting spectra highly subject to noise and interference which reduces the quality of the data and impairs its subsequent analysis providing inconsistent and unreliable results.

9.7.3 Defining copper status

One of the key questions raised in Section 1.12 was the issue of accurate diagnosis for copper status and thiomolybdate presence (Section 1.13). It was discussed how the current methods provided no single measure which could be reliably used as a single indicator of either copper status and/or thiomolybdate presence. For this reason the current work used a combination of methods, which are recommended in practice. However, the use of blood parameters was found to be ineffective in both *in vivo* trials (discussed in Sections 5.5.2 and 8.5.3), as any treatment effects were masked by individual variation. Despite the lack of blood evidence, the results gained from lixiviation gave some interesting insights into the proportion of copper in faeces which remained water soluble. Further research from soil chemistry has highlighted the potential application of using EDTA; which is used for the removal of copper contamination from soil (Lo and Yang, 1999; Kim *et al.*, 2003; Tsang *et al.*, 2007; Ding *et al.*, 2013; Mohamed and Al-Qahtani, 2017), as a potential indicator of thiomolybdate presence. EDTA is a strong chelator for copper; able to strip it from the majority of other ligands, but once bound to thiomolybdate, copper cannot be removed by EDTA; which was supported in the present work in Chapter 8 and in previous studies (Quagraine, 2002; Essilfie-Dughan, 2007). The addition of EDTA to a sample should bind to available copper and solubilise it, allowing for the extraction of copper which is 'free' or bound to non-thiomolybdate ligands, thus leaving only the insoluble copper-thiomolybdate in the sample. EDTA extraction should be able to work on a variety of biological samples and could be used as a diagnostic tool to identify a thiomolybdate problem. Since high molybdenum does not directly equate to thiomolybdate formation, analysis which only detects total elemental concentration is unable to differentiate molybdate from thiomolybdate and TCA extraction will only precipitate in the presence of albumin (Gould and Kendall, 2011). A comparison between total elemental concentration and a calculation of the loss that cannot be extracted by EDTA could account for copper-thiomolybdate formation, but this requires validation. Validation of this technique could be achieved through analysis and comparison of samples using a biological matrix, such as digesta or faeces, specifically created with known concentrations of cuprous-thiomolybdate, sodium molybdate and soluble ionic copper as a control.

9.7.4 The effect of pH

The pH has been documented to have a notable effect on the chemistry of copper compounds (Emanuele and Staples, 1994) (discussed in Section 3.1.3). Throughout this work ruminal pH of ~6 was used in *in vitro* models representative of a grazing ruminant and forage based diets were used in all of the *in vivo* work. However, pH was not measured in the work conducted in Chapters 5, 7 and 8 until after the samples had been collected and through a freeze-thaw cycle. This did not yield appropriate results and could not be included in the analysis. The pH of the rumen may have a significant role in the affinity and progression of the copper-antagonist pathways. Copper availability has been previously reported to change with ruminal pH (Crosby *et al.*, 2004; Gould and Kendall, 2011). Notable changes occur to the rumen environment (discussed in Section 1.5), even within the healthy rumen range, which may not only impact the chemical environment but the presence and function of the bacterial population. Cereal, concentrate based diets promote lactic acid producing bacteria which lower the rumen pH resulting in the decline of the ruminal protozoa population; the importance of which was highlighted in the work of Ivan, Veira and Kelleher, (1986), and Ivan, (1988), discussed in Section 1.6. The increased acidity of the rumen has been proposed to have a beneficial effect on copper availability (Emanuele and Staples, 1994; Crosby *et al.*, 2004; Amat *et al.*, 2014). However, where rumen pH reaches less than 6.5 the conditions promote the formation of tetra-thiomolybdate which has the highest affinity for copper of all thiomolybdate species, discussed in Section 1.7 (Gould and Kendall, 2011). The lower rumen pH from cereal, concentrate based diets has also been shown to increase sulphide production which could contribute to both thiomolybdate and sulphide based antagonisms in the rumen (Kung *et al.*, 2000; Amat *et al.*, 2014; European Food Safety Authority, 2016). Suggesting that the increase in copper availability from a concentrate diet may only be of benefit where molybdenum concentration is negligible. Current research from within the laboratory group supports this hypothesis. In a mineral audit of beef finishing farms the data has indicated that steers fed concentrate diets have disproportionately more thiomolybdate related problems than their pasture fed counterparts despite similar elemental intakes (R. A Schofield and N. R Kendall 2019, personal communication, 22 August), and in an earlier trial, hay fed sheep had greater copper repletion despite feeding elevated molybdenum and sulphur, in comparison to sheep fed fresh herbage or a semi-purified diet (Suttle, 1983). Contrary to this, others have suggested that the resultant increase in sulphide may be absorbed and thus unavailable for interaction and that the lowered pH of the rumen may be sufficient to break down thiomolybdate complexes, while simultaneously increasing copper availability and thus, absorption (Suttle, 1986, 1991, 2010); providing an overall increase in copper absorption and mitigation of thiomolybdate antagonism. This has been evidenced in cattle fed maize silage alongside high molybdenum and sulphur, where no changes in liver copper concentration were observed (Hussein, 2017), but when grass silage was fed alongside high molybdenum and sulphur liver copper concentration decreased markedly (Sinclair *et al.*, 2013). A recent study also reported that liver copper status was higher in cattle fed maize silage in comparison to grass silage, and when molybdenum and sulphur were introduced to the diet the grass silage fed cattle had a greater depletion in liver copper concentration (Sinclair *et al.*, 2017). The reduced comparative copper absorption observed by these workers in fibrous, forage based diets has been attributed to the increased fermentation and residence time in the rumen increasing the time-frame for thiomolybdate binding in the rumen to take place (Suttle, 1991).

There are also potential changes to pH in the omasum. The omasum lacks salivary bicarbonate which in the rumen buffers VFA production. However, VFA absorption in the omasum prevents the pH from falling much beyond the rumen; tending to achieve a pH ~0.5-0.7 lower in the omasum (Prins *et al.*, 1972). In light of a potential new role for this digestive region in copper digestion this change should not be overlooked. The lowered pH is likely to increase the bioavailability of copper, but may also promote the formation of the more copper-potent thiomolybdate species (Gould and Kendall, 2011). Which could potentially promote a second region for thiomolybdate conversion and copper binding, which due to its insoluble nature, is retained within the omasal lining until digestive contractions slowly move it through with the rest of the digesta. Therefore, any further work should incorporate pH measurement to give an insight into its effects on the antagonist pathways.

9.8 Summary of findings

The present research found that considerable changes take place to copper within the rumen (Figure 64). The strong reducing environment in combination with the competing ligands likely results in the full speciation and reduction of copper from copper (Cu^{2+}) chloride. The speciation of simple ionic sources was supported by the feed trial evidence which suggested that not only copper chloride but also TBCC and copper EDTA were rumen soluble sources, and as a result were also subject to changes; some of which resulted in interactions with the antagonists (Figure 64). Further changes also appeared to occur in the omasal-flow leading to the accumulation of copper in this region in the form of an accumulation of copper and iron and continued solubility of TBCC where copper EDTA and copper chloride appeared to have interacted (Figure 64). The present work also showed that the copper in the jejunum changed in its local bonding from the rumen. There was also evidence in the faeces which supported the presence of thiomolybdate and suggested that TBCC might be more resistant to the antagonists effects than the other copper sources tested (Figure 64).

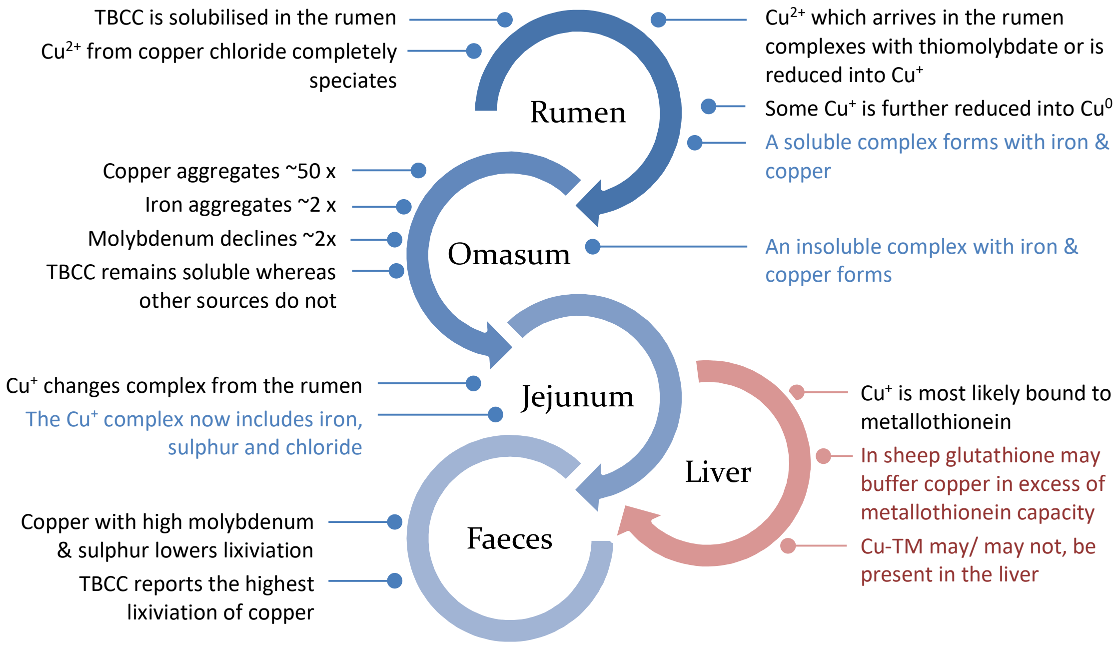

Figure 64: Overview of the key findings throughout the work. Blue/red text denotes hypothetical findings.

9.9 Conclusion

The copper conundrum remains a complex and multi-factorial issue. The present work has made some major advances (Figure 64) in relation to our understanding. However, further investigation of the questions raised throughout this work are required to gain greater insight into the movement of copper through digestion, the formation of the iron-copper-sulphur complex and the interaction of different copper sources with antagonists.

Improving our understanding of the nature of copper's movement through digestion and how this is influenced by the different antagonists will be beneficial for farmers, nutritionists and vets when investigating safe supplementary levels and sources with the aim of providing optimal copper nutrition for ruminants.

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11.0 Appendix I

Welcome

This survey aims to gather information on the current understanding and use of copper supplementation across a wide range of farming enterprises in the UK.

Any information you provide will be anonymised and securely stored. Personal data will not be required for the purposes of this survey, and information relating to geographical location will only be reported as generalised and anonymised data. Individual data and answers will not be reported.

The results of this survey will be used to inform the farming industry, as well as vets, researchers and nutritional advisors of the current understanding in copper supplementation. With the intention that future guidance and information in this area can be better targeted and presented.

❑ *I have read and understood the information provided with this survey and agree to the terms of participation

❑ *I am over 18 years of age

Thank you for undertaking this survey into copper supplementation in farming.

This survey is looking at current understanding, perceptions and practices- there are no wrong answers!

Please give your answers in relation to copper supplementation only, ignoring any other minerals or supplements you may give, and please be honest, and don't look anything up- we are interested in what you already know and think.

Section 1: Animals & Husbandry

Q. Do you personally have a role in the nutritional decisions of your livestock?

☐ yes ☐ no

Q. Do you currently, or have you previously kept pigs on your holding?

☐ no ☐ yes, currently ☐ yes previously, but not now

Q. *Do you currently keep dairy cattle/ beef cattle/ sheep?

☐ yes ☐ no

If 'yes'

Q. What is the total number of stock you keep?

Q. What breed(s) make up your herd/flock? (please give rough percentages of each breed)

Q. During an average year when would the majority of your cattle/sheep be housed without access to pasture?

Q. *In a typical year, do you supplement any of your cattle/sheep with copper?

☐ yes ☐ no ☐ I supplement, but not specifically for copper

If 'yes'

Q. In a typical year, which of the following copper supplements do you use for your cattle/sheep and how often?

Mineral lick (salt lick) /Drench/ Bolus/ Injection/ Pasture dressing/ Mineral feed mix

☐ Never ☐ Once per year ☐ Twice per year ☐ 4 times per year ☐ Every 2 months
☐ Every month ☐ Several times a month ☐ Weekly ☐ Daily

Other, please state supplement type and frequency

Q. Do you differ your supplementation strategy by production stage?

☐ yes ☐ no

If yes, briefly explain how

Q. How relevant are the following statements in your decision to supplement copper to your cattle/sheep?

"I have always supplemented copper"
"Using my current supplementation I have never had any problems with copper"
"I am trying to remedy a fertility problem with no clear cause"
"I am trying to remedy a health problem with no clear cause"
"I received professional nutritional advice"
"I received veterinary advice"
"I received advice from a supplement manufacturer or sales rep"
"I decided to supplement after reading an article in a trade magazine"
"I have observed clinical signs of deficiency in my animals"

"I only supplement copper when the animals are housed"
"I only supplement copper when the animals are at pasture"

❑ not applicable ❑ not relevant at all ❑ low relevance ❑ some relevance ❑ quite relevant

❑ very relevant ❑ this is my main reason

If you have a different main reason, please state

Q. Is your decision to supplement based on any of the following?

Soil analysis/ Herbage, silage, grass or forage analysis/ Blood samples/ Liver samples/ Other tissue samples (eg kidney)/ Other samples (eg milk or urine)

❑ yes ❑ no

Q. If you stopped copper supplementation what % of your animals do you think would have low or deficient copper status?

If 'no' OR 'I supplement, but not specifically for copper'

Q. How relevant are the following statements in your decision not to supplement copper to your animals?

"My pasture meets their copper requirement"
"I feed a TMR which meets their copper requirement"
"I have never observed any signs of deficiency"
"I am worried about toxicity"
"It is too costly"
"I do not have enough labour available"
"I only supplement copper when the animals are housed"
"I only supplement copper when the animals are at pasture"
"It is included in my multi-mineral supplement"
"I used to supplement copper, but don't now"

❑ not applicable ❑ not relevant at all ❑ low relevance ❑ some relevance ❑ quite relevant

❑ very relevant ❑ this is my main reason

If you have a different main reason, please state

If you previously supplemented copper but don't now, please explain why you stopped

Section 2: Copper terminology

Q. Do you feel under pressure to supplement your animals with copper?

❑ I feel under no pressure at all ❑ I feel under very little pressure ❑ I feel under some pressure ❑ I feel under a lot of pressure ❑ I feel under immense pressure ❑ I don't know

Q. Who do you think applies the most pressure to supplement copper?

Family/ Farm workers and staff/ Neighbouring farms/ Magazines or adverts/ Nutritional advisors/ Vets/ Someone else/ Not applicable

❑ Not applicable ❑ No pressure at all ❑ Low pressure ❑ Some pressure ❑ Quite pressuring ❑ Very pressuring ❑ This is the main pressure

If you answered someone else, please give details

Q. In your opinion how accurate are the following statements:

“Copper problems are caused by….”

“….the formation of insoluble copper during digestion"
“….high iron in the total diet"
“….high zinc in the total diet"
“….too much copper in the total diet"
“….a thiomolybdate toxicity"
“….insufficient copper in the total diet"
“….dietary elements causing copper lock-up"
"….thiomolybdate absorption"
"….grazing animals on 'teart' pastures"
"….interactions between copper, iron and/or molybdenum and sulphur from the diet"
"….interactions between copper and manganese from the diet"
"….feeding copper with a low bioavailability"

❑ Totally inaccurate ❑ Partly inaccurate ❑ Neither accurate or inaccurate ❑ Partly accurate ❑ Totally accurate

Q. In your own words without looking anything up, please define the following terms:

'Copper lock-up'
'Primary copper deficiency'
'Secondary copper deficiency'
'Thiomolybdate'
'Molybdenum'
'Molybdenosis'
'Copper-loaded'

☐ I have not heard this term before ☐ I have heard of this term but I don't know what it means
☐ I think I know this term (my definition is below)

Definition:

Q. What percentage of animals in the UK do you think are affected by the following:

Dairy cattle with copper status above normal?
Beef cattle with copper status above normal?
Sheep with copper status above normal?
Dairy cattle with copper status below normal?
Beef cattle with copper status below normal?
Sheep with copper status below normal?

Q. How do you feel about the following statement?

"Cattle are more tolerant of high copper than sheep"

☐ I agree completely ☐ I agree a little ☐ I neither agree or disagree ☐ I disagree a little
☐ I disagree completely

Q. How well do you feel you understand copper related problems?

☐ Not at all ☐ Very little understanding ☐ Some understanding ☐ Sufficient understanding
☐ Good understanding ☐ Excellent understanding

Section 3: Demographic data

Q. To allow classification of your geographical area, please give the first part of your postcode

Q. In years, how much farming experience do you have?

Q. Would you like to learn more about copper problems and supplementation?

❑ yes ❑ no

Q. If you would like to be entered into our prize draw for the free farm mineral audit please leave your email address

Thank You!

for taking the time to complete this survey, your answers are important to us.

Data will be anonymised and collated for analysis and the information you provide will help us immensely with our quest to make copper supplementation easier.

If you have any questions please feel free to contact andrea.clarkson@nottingham.ac.uk