Harper Adams University

A Thesis Submitted for the Degree of Doctor of Philosophy at

Harper Adams University

Optimising the health and performance of dairy cows through improved copper nutrition

James McCaughern

BSc Agricultural Technology (Queen's University Belfast, 2016)

Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

November 2019

Declaration

I declare that this thesis has been originally composed by the author and has not been submitted in a previous degree application. Sources of information used have been acknowledged, as well as work that has been presented at international conferences.

James Hugh McCaughern

November 2019

Acknowledgements

Firstly, I would like to acknowledge the funding of the Agriculture and Horticulture Development Board without which these studies would not have been possible. I would also like to extend gratitude to my supervisory team of Professor Liam A. Sinclair and Doctor Alexander M. Mackenzie for their knowledge, support, and enthusiasm throughout. One for the provision of the carrot, and one for the application of the stick.

I would also like to thank the Ruminant Technical team for their assistance with feeding and sampling, especially for that of Sarah Williams and Carrie Gauld without whom it would not have been accomplished. To the farm staff, thank you, for putting up with my requests for what turned out to be years in the end. I would also like to acknowledge the help of all the students involved over the years including Ella Forrest, Jacob Williams, Paddy Denny, Rowan Boardley, Sophie Wilson, Holly Bowen, Christina Gale, James Bryant, Connor Fettes, Beth Gardner, Hannah Bedford, and Caitlin Fairclough.

To my family, I would like to thank you for your support throughout, we've seen our fair share of suffering in this world. To the newest member, my fiancée Emma Taylor thank you for keeping me going throughout this last year.

Finally, in loving memory of the woman who raised me. Charlotte Lottie McCaughern.

"Trust in the Lord with all your heart and lean not on your own understanding but in his." Proverbs 3: 5-6.

James McCaughern

List of conference abstracts:

McCaughern, J.H., Mackenzie, A.M. and Sinclair, L.A. 2019. Dietary starch level alters rumen pH and copper metabolism in lactating Holstein-Friesian dairy cows receiving added dietary sulphur and molybdenum. *Advances in Animal Biosciences: Animal Science Fit for the Future,* 10, pp. 75.

McCaughern, J.H., Mackenzie, A.M. and Sinclair, L.A. 2019. Dietary starch level alters blood indicators of copper status in lactating Holstein-Friesian dairy cows when fed without or with additional sulfur and molybdenum. *Abstracts of the 2019 American Dairy Science Association Annual Meeting,* 102, pp. 278.

List of Abbreviations:

ADG	Average daily gain
ADF	Acid detergent fibre
ARC	Agricultural Research Council
AOAC	Association of Official Analytical Chemists
As	Arsenic
AST	Aspartate aminotransferase
Atox 1	Antioxidant 1
ATP	Adenosine triphosphate
ATP7A	Adenosine triphosphate 7A
ATP7B	Adenosine triphosphate 7B
BHB	β-hydroxybutyrate
Ca	Calcium
CCO	Cytochrome c oxidase
CCS	Copper chaperone for superoxide dismutase
Cl	Chlorine
Co	Cobalt
COMMD1	Copper metabolism MURR1 domain protein 1
CSIRO	Commonwealth Scientific Industrial Research Organisation
Cox 17	Cytochrome c oxidase copper chaperone 17
CP	Crude protein
CPT-1	Carnitine palmtoyltransferase-1
Cp	Ceruloplasmin
Cr	Chromium
Ctr 1	Copper transporter 1
Cu	Copper
$CuSO_4$	Copper sulfate
DM	Dry matter
DMI	Dry matter intake
EE	Ether extract
F	Fluorine
Fe	Iron
GE	Gross energy

GGT	Ɣ-glutamyltransferase
GH	Growth hormone
GLDH	Glutamate dehydrogenase
Hb	Haemoglobin
HCL	Hydrochloric acid
HCT	Haematocrit percentage
HNO_3	Nitric acid
HS^-	Bisulfide
Hp	Haptoglobin
H_2S	Hydrogen sulfide
I	Iodine
IgG	Immunoglobulin G
IGF-1	Insulin-like growth factor 1
IFN-γ	Interferon-γ
ISO	The International Organisation for Standardisation
Lym No	Lymphocyte numbers
ME	Metabolisable energy
MEb	Metabolisable energy balance
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
Mon No	Monocyte numbers
N	Nickel
NaOH	Sodium hydroxide
NDF	Neutral detergent fibre
NEFA	Non-esterified fatty acids
Neu No	Neutral numbers
NF-κB	Nuclear factor kappa light chain enhancer of B cells
NRC	National Research Council
OM	Organic
P	Phosphorus
PAM	Peptidyglycine α-amidating monooxygenase
PEM	Polioencephalomalacia

PPD	P-phenylenediamine
Na	Sodium
RBC	Red blood cells
S	Sulfur
SARA	Subacute ruminal acidosis
SCC	Somatic cell count
Se	Selenium
SCFA	Short-chain fatty acids
Si	Silicon
Sn	Tin
TGF-β	Transforming growth factor β
TH	Thymus helper cell
TM	Thiomolybdate
TMR	Total mixed ration
V	Vanadium
VLDL	Very low density lipoproteins
WBC	White blood cell counts
Zn	Zinc

Contents

List of Tables

List of Figures

Abstract

The dietary supply of copper (Cu) as an essential trace element has implications for dairy cow performance, health and fertility. Despite being the most widely reported mineral deficiency, there is evidence to suggest an over-supplementation of the element on farm, consequently 38% of Holstein-Friesian cull cow liver that enters the food chain contains toxic liver Cu concentrations. Suggested reasons for this over-supplementation include suppressing the effects of the Cu absorption antagonists sulfur and molybdenum, and a perception from those within the industry that in the absence of clinical toxicity, there are no harmful sub-clinical consequences of elevated hepatic Cu concentrations. Recent evidence however of an increasing Cu status in lactating Holstein-Friesian cows as a result of replacing grass silage with maize silage in the diet, advocates the potential of other nutritional factors to alter Cu absorption. The objective of these studies was to determine dietary factors other than concentrations of Cu antagonists that may have increased hepatic Cu concentration on farm, and determine the long-term consequences of Cu over-supplementation on Holstein-Friesian heifer performance, health, and fertility. Results in study one identify the potential of increased dietary starch concentration and/or decreased ruminal pH to increase the Cu status of lactating Holstein-Friesian cows, and may indicate a lack of understanding within the industry regarding Cu availability in total mixed rations. Despite a marginal growth rate increase in study two, feeding Holstein Friesian heifers Cu in excess of requirement during the growing phase decreased fertility, modulated the immune response, and compromised early lactation performance when continued beyond parturition in study three. In conclusion, increasing dietary starch concentration and/or reducing rumen pH increases hepatic Cu reserves, and the long-term feeding of Cu above requirement during the rearing phase and early lactation may compromise growing heifer fertility, as well as lactation performance.

CHAPTER 1: General Introduction

Copper (Cu) is a transition element with at atomic weight of 63.546 daltons (Georgievskii *et al.*, 1982), it is also an essential trace element in dairy cow diets, and its dietary supply has implications for lactating dairy cow performance, health, and welfare (Suttle, 2010). A deficiency in Cu, otherwise known as a Cu responsive disorder presents with symptoms such as anaemia and impaired growth (McDowell, 1985; Suttle *et al.*, 1987), and can either result from a simple lack of Cu within the diet (Phillippo *et al.*, 1987a), or an interaction with Cu absorption antagonists such as iron, sulfur, or molybdenum (Phillippo *et al.*, 1987b). Despite being the most widely reported mineral deficiency (AHVLA, 2014), recent evidence suggests a large proportion of Holstein-Friesian cull cow liver entering the food chain within the UK contains elevated liver Cu concentrations (Kendall *et al.*, 2015), and may pose a risk to human health (Fukuda *et al.*, 2004). There is evidence of Cu over-supplementation within the industry, surveys in both the United States and United Kingdom have reported Cu supplementation in dairy rations to be in excess of nutritional guidelines (Sinclair and Atkins, 2015; Castillo *et al.*, 2013). Despite this evidence, a large number of British dairy herds continue to supply excess copper (Jacklin, 2016). One of the reasons hypothesised to be responsible for this over-supply of dietary Cu is a lack of understanding regarding the antagonism of Cu in the rumen by sulfur and molybdenum (Kendall *et al.*, 2015). Equations have been developed to try and estimate the effects S and Mo upon Cu absorption (Suttle and McLauchlin, 1976), however these were developed in the 1970's using semi-purified diets in sheep (Suttle and McLauchlin, 1976; Suttle, 2016), and do not consider other dietary factors that may influence Cu absorption such as basal forage type (Hussein, 2017; Sinclair *et al.*, 2017). Studies examining the effects of chronic long-term Cu over-supplementation within dairy cattle are scarce (Suttle, 2010), although evidence is mounting to suggest harmful consequences of dietary Cu loading including impaired rumen function, decreased liveweight gain, and impaired rumen function (Arthington, 2005; Engle and Speers, 2000; Hunter *et al.*, 2013). There is also anecdotal evidence from practicing vets to suggest immunological effects of elevated hepatic Cu concentrations (Howie, 2017). The objectives of this thesis are to determine the mechanisms by which forage type affects Cu availability, and the effects of long-term Cu over-supplementation on Holstein-Friesian health, performance, and fertility throughout the production cycle.

CHAPTER 2: Literature Review

2.1 Introduction

There are at least twenty-two elements that are thought to be essential to animals in order to maintain life (Underwood, 1977). These minerals can be classified as either major (g/kg of DM) or trace (mg/kg of DM) elements depending upon their required concentration within the diet (Underwood, 1977; Underwood, 1981; Table 2.1). There is some evidence to suggest growth and health benefits associated with the supplementation of other elements such as boron, aluminium, cadmium, lithium and lead but these are not classified as essential (Underwood and Suttle, 1999).

Table 2.1. Essential macro and major elements.

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

(Underwood, 1977)

Copper is an essential trace element, and its supply has important implications for the performance and health of lactating dairy cattle (Suttle 2010). Clinical Cu deficiency presents with symptoms exemplified by growth impairment and anaemia (Suttle *et al.*, 1987; McDowell, 1985). There have been a number of studies that have identified increased fertility as a result of appropriate levels of Cu supplementation if cows are deficient (Black and French, 2004; Mackenzie *et al.*, 2001). There is evidence however that high dietary Cu concentrations can cause liver degeneration associated with a reduction in liver function that may impact upon an animal's health, fertility and performance (Suttle, 2010; Laven and Livesey, 2005). Recent surveys report excessive dietary Cu supplementation levels in both

the UK and US (Sinclair and Atkins, 2015; Castillo *et al.*, 2013), which may be partly due to a lack of understanding regarding the effects of antagonists on Cu absorption, especially with respect to sulfur (S) and molybdenum (Mo). (Kendall *et al.*, 2015). This review will discuss Cu metabolism, the difficulties associated with measuring Cu status, and the interactions between Cu and dietary antagonists such as S, Mo, and iron (Fe) in ruminant animals. It will also discuss the potential effects of dietary Cu inclusion on ruminant performance, health, and fertility.

2.2 Chemical and physical properties of copper

Copper is a d-block transition element with an atomic weight of 63.546 daltons and an atomic number of 29 (Georgievskii *et al.*, 1982). The element is found in the environment as one of the three following oxidative states; cupric ion (Cu^{2+}), cuprous ion (Cu^{1+}), or copper metal (Cu^{0}; Georgievskii *et al.*, 1982). The cuprous state readily oxidises to form the cupric state and therefore Cu is predominantly found in the cupric state in most compounds (Linder, 1991; NRC, 2005).

2.3 The main functions of copper

2.3.1 Respiration

Cytochrome c oxidase (CCO) contains three Cu atoms (Horn and Barrientos, 2008), and acts as the terminal electron acceptor in the respiratory transport chain, it is responsible for energy generation in tissues (Suttle, 2010). Low CCO activity has been related to a variety of dysfunctions including impaired respiratory burst in neutrophils (Jones and Suttle, 1987). In cattle succumbing to Cu responsive disorders, one of the earliest pathological lesions is membrane breakdown in cells of the pancreas, where decreased CCO activity may have secondary effects on sulfation and glycosylation (Fell *et al.*, 1985). Gene deletion studies in laboratory mice have shown that animals can’t survive without cytochrome c oxidase (Prohaska, 2006).

2.3.2 Antioxidant

Copper may act as an antioxidant and prevent tissue damage from free radicals produced during processes such as respiration (Birben *et al.*, 2012). The Cu-dependent enzymes superoxide dismutase and ceruloplasmin (Cp) have been shown to scavenge and dispose of superoxide free radicals produced during the neutrophilic respiratory burst (Saenko *et*

al., 1994), a process in which neutrophils kill invading pathogens (Suttle, 2010). Studies conducted on Cu-deprived sheep and cattle have subsequently identified both compromised blastogenesis and phagocytic killing capacity (Jones and Suttle, 1987; Xin *et al.*, 1991).

2.3.3 Iron transport

Hephaestin is a Cu-dependent enzyme with 50% homology to ceruloplasmin (Section 2.8.1), with similar properties such as ferroxidase activity (Suttle, 2010). Hephaestin is required for efficient efflux of Fe across the basolateral membrane of the enterocyte and subsequent incorporation into Fe-transferrin for transport to the liver where it is stored (Liu *et al.*, 2007). Copper deficiency has therefore been shown to reduce enterocyte Fe efflux (Collins *et al.*, 2010). In addition to decreased absorption, the absence of Cp has been shown to cause Fe accumulation in the brain, pancreas, and retina, indicating that the enzyme is vital for Fe homeostasis within the animal (Xu *et al.*, 2004). Erythroid cells as the precursors of erythrocytes are the most avid consumers of Fe within the body (Collins *et al.*, 2010). It is therefore not surprising that an anaemia has been observed in Cu-deficient cattle and sheep (Barry *et al.*, 1981; Suttle *et al.*, 1970).

2.4 Copper transport within the ruminant

Copper metabolism within an organism involves absorption, transport, storage, utilisation and excretion of the element in order to maintain life's biological processes (Kalinowski, 2016). A number of authors who have utilised stable isotopes to determine the quantitative movement of copper within ruminants (Buckley, 1991; Weber *et al.*, 1983). Weber *et al.* (1980) identified three main pools of Cu in sheep via the use of ^{64}Cu. Buckley (1991) was able to demonstrate Cu metabolism in dairy cattle in much greater detail using ^{65}Cu. The author identified that approximately 5.1% of dietary Cu is absorbed of which 92% reaches the liver (Buckley, 1991).

2.4.1 Absorption

It was previously thought that Cu absorption was concentrated in the duodenum of most mammals (Evans, 1973). Ruminants however were hypothesised to differ regarding their absorption physiology at this time (Turner *et al.*, 1987). Turner *et al.* (1987) reported that in sheep a large proportion of the alimentary tract seemed to be capable of Cu absorption including the abomasum, small intestine and colon, however absorption was not observed

from the reticulum or rumen itself. The main criticism levelled at a lot of this historical work is that it was developed by analysing Cu disappearance from the gut (Cousins, 1975; Evans, 1973; Turner *et al.*, 1987), whereas modern day molecular techniques identify that Cu is absorbed in the small intestine (Kalinowski, 2016; Vonk *et al.*, 2008).

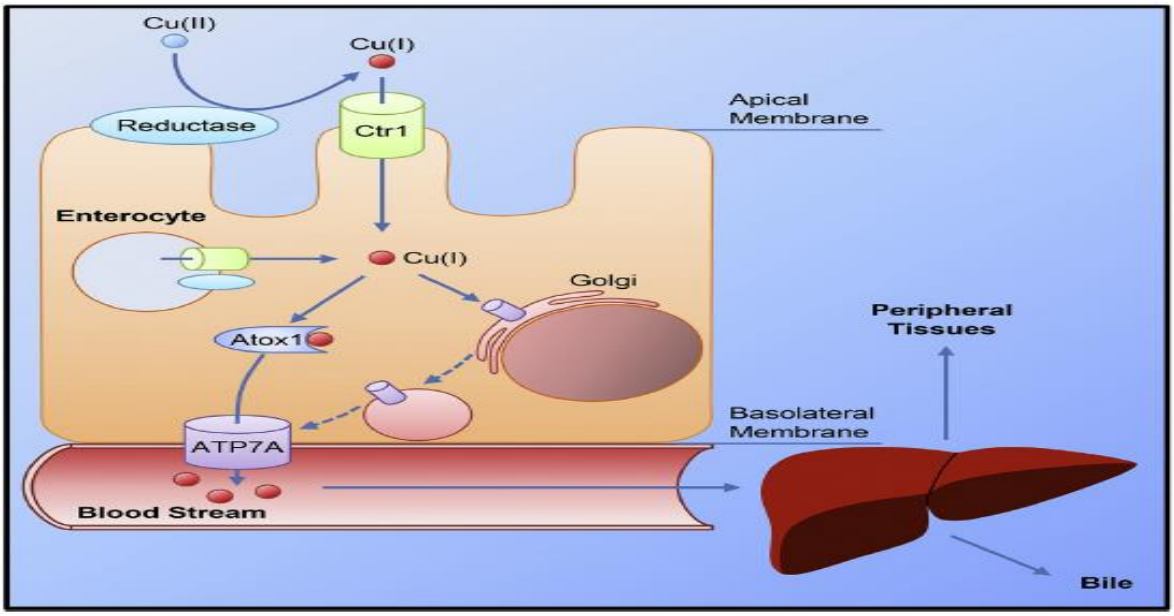

Figure 2.1. Enterocyte Cu absorption (Kalinowski, 2016).

Cu^{2+} is reduced to Cu^{+} by a brush border metalloenzyme at the apical surface before being transported across the apical membrane of the enterocyte by the high-affinity Cu^{+} transporter Ctr 1. Cu can then be transported into the trans-golgi network for the synthesis of metallo-enzymes and/or be shuttled by Atox 1 to ATP7A in order to be pumped into the blood stream.

The mechanisms by which Cu is transported into enterocytes from the gastrointestinal tract are thought to vary according to dietary Cu concentration (Kalinowski, 2016), whereby Cu passes into the enterocyte by passive diffusion at high dietary Cu levels, whilst facilitated diffusion mediated by a saturable carrier is preferred at low dietary Cu concentrations (Linder and Hazegh-Azam, 2006). Copper transporter 1 (Ctr1) has been identified as the primary transmembrane protein responsible for transporting Cu into the enterocyte in the Cu^{+} form (Harris, 2000; Lee *et al.*, 2002; Wee *et al.*, 2013). Dietary Cu however predominantly exists in the Cu^{2+} form, and so must be reduced prior to absorption, this reaction can be catalysed by reductases already present in the diet and/or those produced by the enterocyte itself (Kidane *et al.*, 2012; Lee *et al.* 2002; Figure 2.1). Once in the cytoplasm of the enterocyte Cu can enter the bloodstream via a number of pathways. It can be transported to the Golgi network and used for the production of Cu dependent enzymes (Kim *et al.*, 2008), alternatively it can be shuttled across the enterocyte by the Cu chaperone

antioxidant 1 (Atox 1; Kalinowski, 2016). These pathways have a common end stage involving the transport of Cu into the portal circulation across the basolateral membrane by adenosine triphosphatase 7A (ATP7A; Kim *et al.*, 2008).

Copper absorption efficiency varies between species, with ruminants having little control over absorption and tend to store excess Cu, whilst non-ruminants regulate absorption to a greater extent allowing them to maintain lower liver Cu concentrations (Suttle, 2010). Ruminants of the same species may have differing apparent Cu absorption efficiencies as a result of a number of factors including; animal age (NRC, 2001; Suttle 1975), breed (Ward *et al.*, 1995), dietary form of copper supplied, gestational status (Vierboom *et al.*, 2002), stress (Nockels *et al.*, 1993), and the presence of dietary antagonists (Section 2.5). For instance, the Cu status of Jersey cows will increase at a higher rate relative to that of Holsteins when fed a similar dietary Cu concentration (Du *et al.*, 1996). Ward *et al.* (1995) also identified lower rates of Cu absorption in Simmental steers relative to Angus steers. There is also evidence to suggest increased Cu absorption by cattle during pregnancy (Vierboom *et al.*, 2002), whilst Nockels *et al.* (1993) identified decreased Cu retention in steers stressed by the administration of adrenocorticotropic hormone. NRC (2001) also reported differing Cu requirements for dairy cattle at different stages of development, whilst Suttle (1975) observed that Cu availability decreased from 74.2% pre-weaning to 10.8% post-weaning when artificially reared lambs were dosed with a labelled Cu isotope (^{64}Cu).

2.4.2 Transport and distribution

Once Cu enters the portal circulation, it is transported to the liver primarily bound to serum albumin, with transcuprein and histidine representing other potential transportation pathways (Linder *et al.*, 1998; Valko *et al.*, 2005). The liver is the main organ of Cu homeostasis within the body, and acts as a storage organ, regulates Cu excretion, and secretes Cu into the blood for transport to other bodily tissues (Mercer and Llanos, 2002).

Copper is partitioned three ways when it reaches the liver; storage in the liver itself, utilised for the formation of ceruloplasmin, or excreted in bile (Bremner, 1993). The Cu transporter Ctr1 is again responsible for Cu uptake from the portal circulation into the hepatocyte, where copper chaperones act to deliver the absorbed Cu to various compartments within the cell (Markossian and Kurganov, 2003; Tapiero and Townsend, 2003; Figure 2.2). These Cu chaperones have the following functionality:

- Atox1 - transports Cu to the transporting ATPases (ATP7A/ATP7B; Lutsenko *et al.*, 2007).
- Copper chaperone for superoxide dismutase (CCS) - directs Cu to the formation of copper/zinc superoxide dismutase (SOD) which protects against oxidative stress (Nyasae *et al.*, 2007).
- Cytochrome c oxidase Cu chaperone 17 (Cox17) – transports Cu to the mitochondria within a cell for the formation of cytochrome c oxidase (Leary *et al.*, 2004).
- Glutathione- directs Cu to the formation of proteins involved in the detoxification of metals on an intracellular basis and can also donate Cu to Atox 1 (Wang and Guo, 2006).

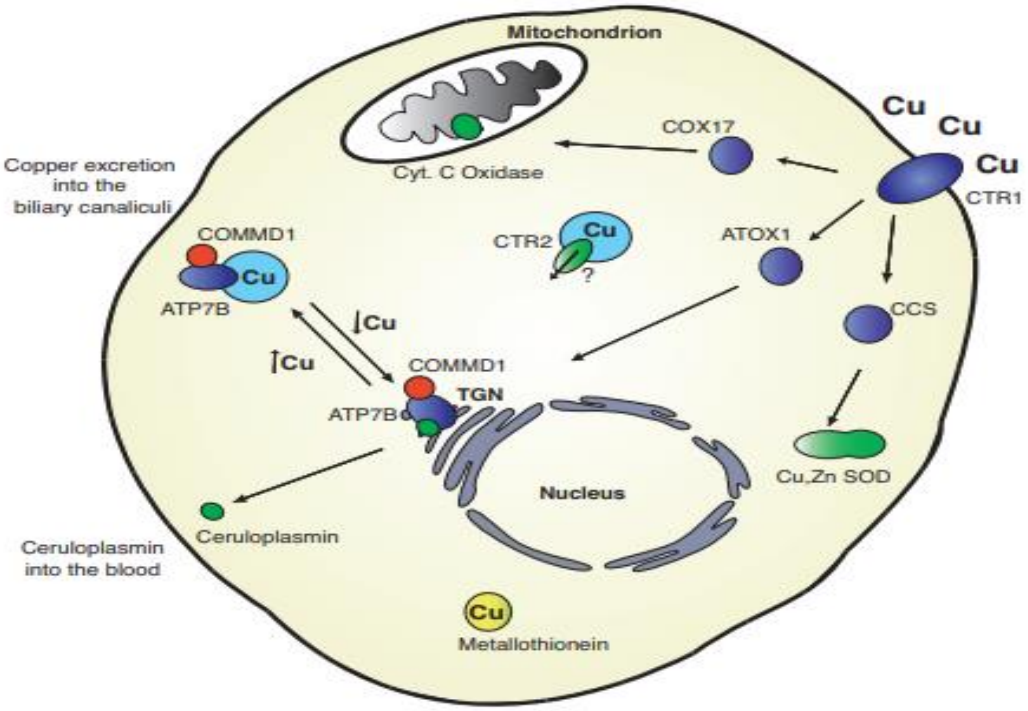

Figure 2.2. Copper uptake and distribution in the hepatocyte (Kalinowski, 2016; Vonk *et al.*, 2008).

Cu uptake into the hepatocyte is mediated by Ctr1. Following absorption Cu binds to several cytosolic Cu chaperones for intracellular transport including; CCS for delivery to Cu/Zn SOD_1, Cox 17 for delivery to cytochrome c oxidase, and Atox 1 for transport to the trans-golgi network where ATP7B incorporates Cu into ceruloplasmin. Free intracellular Cu is stored in vesicular Cu pools or bound to metallothioneins. Ctr2 may have a role in releasing Cu from these storage pools. In cases of excess Cu supply to the cell, ATP7B translocates to the cell membrane of the cell and promotes the transport of Cu from the cell into the bile. COMMD1 has been hypothesised to co-operate and regulate the function of ATP7B.

Ceruplasmin (Cp) is the primary transport mechanism of Cu around the body (Kidane *et al.*, 2012). Despite this, evidence exists to suggest other mechanisms of transport as mice can still transport sufficient Cu when the gene which codes for Cp is absent (Hellman and Gitlin, 2002).

The transporter ATP7B is predominantly found in the liver where its roles involve; directing Cu to the trans-golgi network, directing Cu into the Cp pool, and the excretion of Cu into the bile (Harris, 2000). Copper metabolism MURR1 domain protein 1 (COMMD 1) has been hypothesised to regulate the function of ATP7B in vesicular Cu sequestration (La Fontaine *et al.*, 2010). Wilson's disease is a type of Cu toxicity that occurs in humans with mutations of the ATP7B gene (Kodama *et al.*, 2012), where Cu can neither be incorporated into Cp nor excreted in the bile (Shim and Harris, 2003), leading to a build-up of hepatic Cu and symptoms ranging from cognitive impairment to hepatic failure (Underwood and Suttle, 1999). The transport protein ATP7A is distributed throughout the body where it directs Cu to the trans golgi-network for the synthesis of metalloenzymes and is also responsible for Cu transport from enterocytes into the blood stream (Harris, 2000). Menkes disease is caused by mutations in the ATP7A gene resulting in Cu deficiency (Chelly *et al.*, 1993; Das *et al.*, 1995; Vulpe *et al.*, 1993). These mutations inhibit Cu absorption in the small intestine, and result in Cu accumulation in the cytoplasm of cells leading to decreased activity of cuproenzymes and Cu deficiency (Kodama *et al.*, 1993; Kodama *et al.*, 2012).

2.4.3 Storage and excretion

The predominate mechanism of Cu storage within the hepatocyte of the ruminant is the large-granule fraction (lysosome-fraction; Lòpez-Alonso *et al.*, 2005), followed by the nucleus and cytosol fraction (Lòpez-Alonso *et al.*, 2005). Microsomal fractions by comparison store small quantities of Cu (Kumaratilake and Howell, 1989; Corbett *et al.*, 1978; Gooneratne *et al.*, 1979). This is in contrast to other species of mammals where under adequate liver Cu concentrations the large-granule fraction may only account for 20% of Cu storage, whilst the cytosol may store in excess of 50% (Evans, 1973; Corbett *et al.*, 1978). It is hypothesised that this difference in Cu distribution between species may be a result of a decreased ability of ruminants to produce metal-binding proteins (Saylor *et al.*, 1980). Hepatocyte Cu distribution may also vary as a result of age and Cu status (Gooneratne *et al.*, 1979; Lòpez-Alonso *et al.*, 2005). The liver itself accounts for a much higher proportion of total copper storage in ruminants relative to other species (Underwood, 1977). Evidence

from adult sheep suggests that the liver represents 79% of total Cu storage in ruminants whenever the animal experiences a high Cu status (Dick, 1954).

The secretion of bile by the liver is an important component of homeostatic control, and it is hypothesised that bile accounts for approximately 80% of all Cu excreted (Kim *et al.*, 2008; Tapiero *et al.*, 2003). ATP7B is responsible for the loading of hepatic Cu into bile (Lenartowicz and Krzeptowski, 2010; Lutsenko *et al.*, 2002). Despite the identification of the ATPases as Cu transporters, their mRNA levels do not appear to vary in accordance with Cu status (Prohaska, 2008; Sinclair *et al.*, 2013). Biliary Cu concentrations however have been shown to increase by a factor of five when liver Cu status increases (Suttle *et al.*, 2002). Cattle will begin to limit liver Cu storage at lower hepatic concentrations in comparison to sheep when fed high dietary levels (Phillipo and Graca, 1983). It is hypothesised that this is a result of lysosomes in cattle having a reduced threshold for Cu storage compared to those in sheep (Lopez-Alonso *et al.*, 2005). There is also evidence that Cu excretion may differ between breeds. Gooneratne *et al.* (1994) reported decreased biliary Cu concentrations in Angus relative to Simmental cattle. Small amounts of Cu can also be excreted in urine, this however accounts for a very small proportion of total Cu excretion and is relatively constant across all species regardless of Cu supplementation level (Smith *et al.*, 1968). Sheep are the exception where there is evidence to suggest increase in urinary Cu excretion as a result of Mo consumption (Smith *et al.*, 1968).

2.5 Copper antagonists

2.5.1 Copper-sulfur metabolic interactions

Sulfur in dietary feedstuffs is present in both organic (S-containing amino acids) and inorganic (mainly sulfates) forms, with typical pasture and conserved forage concentrations of sulfur varying from 0.5 to 5.0 g/kg DM (Suttle, 2010; Spais *et al.*, 1968). In the rumen, S-containing amino acids and/or inorganic sulfates are reduced to hydrogen sulfide (H_2S) by sulfur reducing bacteria and excreted into the ruminal fluid (Bradley *et al.*, 2011). Some of H_2S produced disassociates to form bisulfide (HS^-) in the fluid, whilst the remaining H_2S migrates to the gas cap of the rumen (Schoonmaker and Beitz, 2012). This reaction is pH-dependent, whereby at a rumen pH of 5.5, approximately 5% of H_2S will disassociate to HS^-, whereas at a pH of 7.0, approximately 50% will disassociate to HS^- (Drewnoski *et al.*, 2014a). Sulfide in the rumen fluid is subsequently free to form an insoluble Cu sulfide compound which is suggested to reduce Cu availability (Suttle, 1974), however this antagonistic effect of S in isolation is hypothesised to be minor in comparison to that of the

Cu-S-Mo interaction (Suttle, 1974; Section 2.5.2). Hydrogen sulfide accumulated in the gas cap is subsequently eructated and inhaled (Dougherty and Cook, 1962), where it reaches the brain via the blood stream causing necrosis of the grey matter in a condition known as polioencephalomalacia (PEM; Gould, 1998; Niles *et al.*, 2002). This condition has been associated with decreased growth and DM intake in finishing cattle (Drewnoski *et al.*, 2014a). Threshold ruminal H_2S concentrations that put cattle at risk of S-induced PEM have yet to be determined, although estimates range from 2000 to 12000 mg/L (Drewnoski *et al.*, 2012; Gould *et al.*, 1997).

2.5.2 Copper-sulfur-molybdenum metabolic interactions

Interactions between Cu, S, and Mo have been reviewed by a variety of authors (Suttle, 1991; Gould and Kendall, 2011; Dick *et al.*, 1975). Forage Mo concentrations tend to be low at approximately 1-2 mg/kg DM, however in certain areas of the UK pasture Mo concentrations may exceed 10 mg/kg DM (Suttle, 2008). Evidence of dietary antagonism between Cu and Mo originates from cattle grazing pasture high in Mo which developed a syndrome characterised by anaemia, diarrhoea, and reduced live weight gain (Suttle, 1974). Dietary Mo is readily absorbed (Mills and Davis, 1987), indeed when Mo concentrations are increased in isolation, they appear to have little effect on Cu availability but result in increased plasma Mo (Suttle, 1974). In contrast, when dietary Mo concentrations are increased from 0.5 to 4 mg/kg DM in conjunction with an increase in dietary sulfur from 1 to 3 g/kg DM, Cu availability declined by approximately 50% (Suttle, 1975). This decrease in Cu availability resulting from S and Mo supplementation (Figure 2.3) has been noted by several authors over the years (Suttle, 2010; Sinclair *et al.*, 2013; Sinclair *et al.*, 2017). It is now recognised that dietary sulfides within the rumen react with molybdate to form thiomolybdates, that subsequently form an insoluble complex with Cu preventing its absorption (Suttle, 1991). This reaction is both reversible and pH dependent, and involves molybdate reacting with HS^- in a stepwise fashion whereby there is sequential replacement of 0^{2-} with S^{2-} to form thiomolybdate isomers as shown below:

$MoO_4^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_3S^{2-}$ Monothiomolybdate (TM_1)

$MoO_3S^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoO_2S_2^{2-}$ Dithiomolybdate (TM_2)

$MoO_2S_2^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoOS_3^{2-}$ Trithiomolybdate (TM_3)

$MoOS_3^{2-} + H^+ + HS^- \leftrightarrow H_2O + MoS_4^{2-}$ Tetrathiomolybdate (TM_4)

Clarke and Laurie, (1980); Gould and Kendall, (2011)

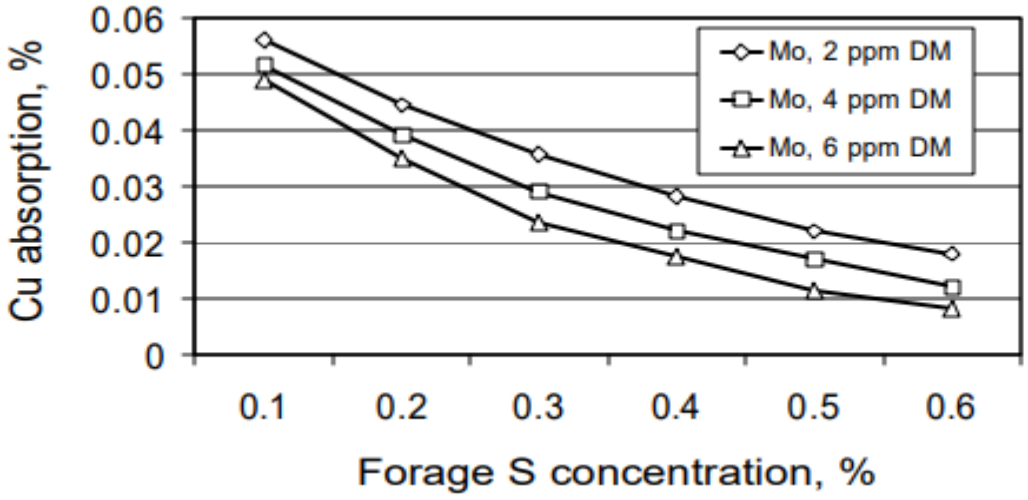

Figure 2.3. Copper absorption efficiency in the presence of varying concentrations of sulfur and molybdenum (Arthington, 2003).

Clarke and Laurie (1980) identified an increased rate of tetrathiomolybdate formation at lower rumen pH values *in vitro*. The authors did note that these findings do not consider pH effects on sulfide supply to the reaction *in vivo*, which is of importance as sulfur cycling within the rumen has the potential to increase or decrease at a much faster rate relative to that of thiomolybdate formation (Clarke and Laurie, 1980). The formation of different thiomolybdate species is hypothesised to have varying consequences regarding Cu absorption and metabolism (Suttle, 1991). The TM_3 and TM_4 species have been suggested to irreversibly bind Cu to high molecular weight proteins decreasing Cu absorption (Suttle, 1991; Suttle and Field, 1983). In contrast, TM_2 was observed to decrease Cu absorption in high sulfur diets with no effect on availability in low sulfur diets, leading to the hypothesis that it was converted to the "higher" (TM_3 and TM_4) species in the high sulfur diets (Suttle and Field, 1983). It is also presumed that TM_1 is broken down in the abomasum freeing any complexed Cu and making it available for absorption (Price *et al.*, 1987). In order to demonstrate any systemic effects within the animal, thiomolybdates must be absorbed (Suttle, 1991), and Mason *et al.* (1982) concluded that under normal conditions TM_3 is the most likely cause of any systemic effects within the animal as it was detected in the plasma of sheep following large ruminal doses of molybdate.

Under the correct dietary conditions as exemplified by high dietary S and Mo, coupled with low levels of dietary Cu, TM_3 and/or TM_4 may enter the bloodstream (Suttle, 1991; Suttle, 2010). When these higher forms enter the portal circulation, they may bind to form a complex with Cu and albumin (Woods and Mason, 1987). The formation of these complexes

in vivo has only been demonstrated following excessive Mo supply made possible by experimental supplementation or intravenous administration of TM_3 and TM_4 (Mason *et al.*, 1986). Once formed, the complex can have important physiological effects including; removal of Cu from SOD during absorption across the intestinal epithelium (Suttle *et al.*, 1990), restricting Cu delivery to the liver via the hepatic portal vein for Cp synthesis (Kelleher and Mason, 1986), an inhibition of the diamine oxidase activity of Cp (Lannon and Mason, 1986), and/or an increase in biliary Cu excretion (Ke and Symonds, 1989). Previously, Cp inhibition was only thought possible at pharmacological doses *in vitro* (Lannon and Mason, 1986), and not even TM_4 had shown inhibition of the enzyme *in vivo* (Suttle, 1991). There is however recent evidence in both sheep and cattle illustrating potential effects of dietary S and Mo inclusion on Cp activity as discussed in Section 2.8.2. (Hussein, 2017; Sinclair *et al.*, 2017; Table 2.9).

The formation of TMs, particularly of higher forms (TM_3 and TM_4) within the rumen is responsible for decreased Cu availability (Gould and Kendall, 2011). Indeed, as the agonist to antagonist (Cu: Mo) ratio within the diet reaches 1:1, it is thought that TM_3 has the potential to be absorbed in sufficient quantities to complex albumin and cause systemic effects (Suttle, 1991). Care however should be taken when using this ratio as an indicator of Cu availability as estimates range from >2:1 for normal growth in beef cattle to 4:1 in order to safely avoid swayback (Alloway, 1973; Miltimore and Mason, 1971). This critical ratio may also be diagnostically imprecise for a variety of reasons including no consideration for dietary sulfur and/or dietary forage diet type (Suttle and Field, 1983). Suttle and McLauchlin (1976) went a step further towards quantifying the Cu x S X Mo reaction using semi-purified diets in the equation below:

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

A = Cu availability (mg/mg)

S = sulfur content (g/kg DM)

Mo = molybdenum content (mg/kg DM)

This equation however does not take into consideration other dietary factors that have the potential to influence Cu availability such as forage type (Suttle, 1986; Table 2.9). Sinclair *et al.* (2017) noted a greater decrease in hepatic copper retention of 1.46 mg/kg DM/d in Holstein Friesian dairy cows fed grass silage- as opposed to a decrease of 0.55 mg/kg DM/d

for cows fed maize silage-based diets supplemented with S and Mo. Indeed, when the equations of Suttle and McLauchlin (1976) were tested under grazing conditions in Canada with the aim of predicting hypocupremia, they proved unsuccessful (Boila *et al.*, 1984).

2.5.3 Copper iron metabolic interactions

In addition to S and Mo, the dietary inclusion of iron has the potential to reduce the Cu absorption coefficient in ruminants (Suttle, 2010; Table 2.2). Iron ingestion normally results from the soil contamination of feedstuffs during practices such as the harvesting or wilting of grass silage (Sinclair and Mackenzie, 2013; Suttle, and Peters, 1985; Standish *et al.*, 1971). Studies comparing the antagonistic effects of Mo and Fe have shown similar initial reductions in hepatic Cu (Gengelbach *et al.*, 1994; Phillippo *et al.*, 1987a, b). There is little published evidence however that the Cu-Fe interaction on Cu availability may add to the Cu-Mo-S interaction in either cattle or sheep (Suttle, 2010; Humphries *et al.*, 1983).

There are a variety of mechanisms that have been proposed by which dietary Fe may reduce hepatic Cu concentrations (Gould and Kendall, 2011; Hansen *et al.*, 2008; Fry, 2011). The first occurs in the rumen where Fe may react with sulfide and Cu to form an insoluble Fe-Cu-S complex (Gould and Kendall, 2011). Alternatively, sulfide may combine with Fe in the rumen to form iron sulfide, Fe in this complex then exchanges with Cu to form copper sulfide (Gould and Kendall, 2011). The second mechanism occurs in the intestines whereby soluble Fe causes a down-regulation of a non-specific carrier protein thought to be involved in Cu uptake (Hansen *et al.*, 2008; 2010). The final mechanism is hypothesised to occur in the liver where high dietary Fe concentrations induce the transcription of transport proteins involved in Cu efflux into the bile (Fry, 2011).

Table 2.2. The effect of additional dietary iron on liver copper concentrations.

Author	Species	Additional Fe (mg/kg DM)	Study duration (wks)	Initial liver Cu (mg/kg DM)	Final liver Cu (mg/kg DM)
Sefdeen, (2017)	Sheep	800	13	545	274
Sefdeen, (2017)	Sheep	750	6	313	205
Phillippo et al., (1987a)	Cattle	800	32	94.5	3.6
Phillippo et al., (1987b)	Cattle	500	32	134	5.5

2.6 Cu deficiency

Copper deficiency, clinically termed hypocuprosis results from a depletion of hepatic Cu reserves beyond the point where the liver is no longer able to maintain Cu homeostasis within the animal (Suttle, 1987; Suttle, 1991). Susceptibility to the condition varies between species and their stage of deprivation (Suttle, 2010). Copper deficiency can either occur as a result of insufficient dietary supply causing primary deficiency (Phillippo *et al.*, 1987a), or as a result of interactions with one or more Cu antagonists which reduce Cu absorption efficiency causing secondary deficiency (Phillippo *et al.*, 1987b; Section 2.5). It may be more prudent therefore to consider “Cu-responsive disorder” as a more appropriate term to cover both causes of the condition than the use of “Cu deficiency”. It is hypothesised that cattle will begin to exhibit clinical signs of Cu deficiency as summarised in table 2.3 when liver concentrations decrease below a critical threshold of 19 mg /kg DM (Laven and Livesey, 2005).

Table 2.3. Symptoms of clinical Cu deprivation in cattle and sheep.

Symptom	Biological summary	Source
Ataxia (swayback)	• Neurological disorder affecting lambs or kids around birth characterised by staggering.	Ivan *et al.*, (1990)
	• Earliest clinical symptom of Cu deprivation in sheep.	Suttle *et al.*, (1970)
	• Calves do not succumb to neonatal or delayed ataxia.	Suttle, (1987)
Depigmentation	• Greying of black hair or bleaching of brown hair.	Hansen *et al.*, (2009)
	• Earliest warning sing of Cu deprivation in cattle.	Hansen *et al.*, (2009)
Anaemia	• Develops in cases of prolonged or severe deprivation.	Suttle, (1987)
	• Oxidative stress results in Heinz body formation in erythrocytes.	Suttle *et al.*, (1987)
	• Heinz body anaemia has been reported in Cu deficient cattle fed kale.	Barry *et al.*, (1981)
Bone disorders	• Bone abnormalities vary between and within species.	Suttle, (2010)
	• Uneven bone growth due to endochondral ossification.	Mills *et al.*, (1976)
	• Ricket-like lesion in the lower limbs of growing cattle.	Mills *et al.*, (1976)
	• Osteitis fibrosa in Cu deprived calves.	Mills *et al.*, (1976)
	• Beading of the ribs in sheep and cattle.	Cunningham, (1950)
Cardiovascular disorders	• Cardiac lesions were attributed to Cu deprivation in cattle which died suddenly in Western Australia- Falling disease.	
	• This has not been observed elsewhere geographically or in other species.	Bennetts and Hall, (1939)
	• Cardiac enlargement has however been observed in experimentally deprived calves.	Mills *et al.*, (1976)
Infertility	• Delayed oestrus in beef cows grazing Cu deficient pastures.	Phillippo *et al.*, (1982)
	• The relationship with Cu status is inconsistent and maybe associated with Mo supplementation/toxicity.	Phillippo *et al.*, (1987b)
Growth retardation	• Symptom of Cu deprivation in grazing sheep.	Woolliams *et al.*, (1986)
	• Biochemical explanations are unclear.	Suttle, (2010)
	• But appears to be a result of Mo toxicity as opposed to Cu deprivation.	Wittenberg and Devlin, (1987)

2.7 Copper toxicity in cattle

2.7.1 Copper status of the UK dairy herd

The Cu status of the UK dairy industry can be considered a nutritional dichotomy (AHVLA, 2014; Kendall *et al.*, 2015). Despite Cu being the most widely reported mineral deficiency (AHVLA, 2014), a large proportion of liver entering the food chain from dairy culls has been shown to contain toxic or high liver Cu concentrations (Kendall *et al.*, 2015). Fukuda *et al.* (2004) identified an increased risk of a variety of cancers including those of the urinary tract when humans are exposed to elevated levels of dietary copper.

In recent years, several authors have identified over-supplementation of Cu within the UK dairy industry (Sinclair and Atkins, 2015; Kendal *et al.*, 2015). The UK however is not alone in this respect, with the Cu loading of bovine liver being reported in the United States (Castillo *et al.*, 2013). Castillo *et al.* (2013) reported that Cu was being fed at approximately 0.9 times above NRC requirements on 39 Californian farms, with 10% of farms feeding 3 times the recommended dietary concentration. Sinclair and Atkins (2015) identified that 32 out of 50 herds surveyed in Northern and Central England supplemented Cu at a level in excess of the industry guideline maximum of 20 mg Cu/kg DM (ACAF, 2010). Indeed, six of the survey farms were supplementing Cu in excess of the then legal level of 40 mg/kg DM set by EU regulation 1831/2003 on animal feed additives (Sinclair and Atkins, 2015). It has subsequently been identified that approximately 38% of the UK's Holstein-Friesian dairy cows had liver Cu concentrations exceeding the 508 mg/kg DM threshold considered to pose a risk of clinical Cu toxicity (Kendall *et al.*, 2015; Livesey *et al.*, 2002). Despite this evidence of over-supplementation, a considerable number of British dairy herds continue to supply Cu at excessive levels (Jacklin, 2016).

This over-supplementation of Cu may have a variety of causes (Kendall *et al.*, 2015), often it can simply be due to an accumulation of high Cu feed ingredients combined with poor record keeping (Kendall *et al.*, 2015; Johnston *et al.*, 2014). Additionally, there may also be a cultural cause where a "more is better" attitude results in overfeeding (Kendall *et al.*, 2015). Suttle (2016) attributed increasing incidence of bovine Cu toxicity to an "ignorance" of greater Cu availability in total mixed rations (TMR). Indeed, Suttle (2016) called into question the validity of current Cu supplementation recommendations as they were developed using grass-based diets, and hypothesised that the majority of TMRs may need no more than 5 mg Cu/kg DM.

2.7.2 Recommended levels of Cu supplementation

There is some variation in dietary Cu recommendations (Table 2.4), primarily as a result of different absorption coefficients being used in requirement calculations (NRC, 2001; CSIRO, 2007). The mean supplementation level of 27.9 mg/kg DM during early lactation identified by Sinclair and Atkins (2015) on UK dairy farms was 1.8 times greater than the maximum guideline. Indeed, if the Cu absorption coefficient were to halve from a typical value of 0.03 to 0.015, the Cu requirement of a 650 kg early lactation cow according to CSIRO (2007) would increase from 11 mg/kg DM to 22 mg/kg DM, and remain well below the mean level of Cu supplementation received by the UK's winter fed dairy cows (Sinclair and Atkins, 2015).

Table 2.4. Approximate Cu requirements of dairy cattle at different production stages according to various national bodies.

	Dietary copper requirement mg Cu/kg DM			
Livestock Class	ARC[1] (1980)	NRC[2] (1989)	NRC[2] (2001)	CSIRO[3] (2007)
300 kg heifer (Weight gain = 0.7 kg/day)	11.8	10	12	8.6
500kg heifer (day 250 of gestation; Weight gain = 0.5 kg/day)	15.4	10	15.2	13.3
650 kg cow (Milk yield = 40 kg/day)	10.7	10	15.7	11
650 kg cow (day 270 of gestation)	13.9	10	13.7	16.7

[1]Agricultural Research Council (ARC)
[2] National Research Council (NRC)
[3] Commonwealth Scientific and Industrial Research Organisation (CSIRO)

2.7.3 Dietary copper sources

The Cu content of animal feedstuffs varies widely as illustrated by Table 2.5. Pasture Cu content varies due to several factors including; plant species, maturity, soil conditions and the addition of fertilisers (McFarlane *et al.*, 1990). Underwood and Suttle (1999) reported that the Cu content of a pasture can increase from 4.5 to 21.1 mg/kg DM when different grass species are grown on the same soil. Minson (1990) identified that grasses tend to have lower Cu concentrations than legumes when grown under the same conditions.

Cereal grains tend to have low Cu concentrations, with small differences between species (Todd, 1972). Rich sources of Cu within the diet include oilseed and leguminous meals with

concentrations ranging from 25-40 mg/kg DM (Chooi *et al.*, 1988). Distillery by-products can contribute some of the highest Cu concentrations to the diet with levels as high as 86 mg/kg DM reported in pot ale syrup (Suttle *et al.*, 1996).

It should also be taken into consideration that feed sources differ in terms of their Cu availability (Suttle, 2010). The reasons for this difference are not completely clear, however it is thought that different forms of Cu in feeds account for at least some of this difference (Suttle *et al.*, 1996). For example, the Cu availability of grazed grass has been reported to be poor (2.5%; Mo and S content depending) in comparison to cereals (9.1%) and cereal by-products (5.0%) (Suttle *et al.*, 1996).

Table 2.5. The copper content of dietary components.

Feedstuff	Cu content (mg/kg of DM)
Grass (Extensive grazing)	7.0
Grass silage (mature)	3.0
Maize silage	2.5
Lucerne	11.0
Wheat	5.0
Barley	4.8
Soya bean meal	25.0

McDonald *et al.*, (2011)

2.7.4 Clinical copper toxicity

Clinical Cu toxicity can originate from either a single large dose (acute) or an accumulation (chronic) of the element over a period of time (NRC, 2005). Species differ in terms of their ability to cope with dietary Cu. Cattle are thought to be much less susceptible to Cu toxicity than sheep with the acute onset dose estimated at 200 mg Cu/kg of bodyweight for cattle in comparison to 9-20 mg Cu/kg for sheep (NRC, 1980).

Chronic Cu toxicity is most commonly found in lambs fed a high concentrate diet (Underwood and Suttle, 1999). There are two recognised stages of Cu toxicity consisting of a prehaemolytic phase and haemolytic phase (Howell and Gooneratne, 1987). Despite an accumulation of Cu in the liver during the prehaemolytic phase the animal is observed to be clinically normal, and there is no reported detrimental effect on animal performance during this phase (Engle and Spears, 2000; Howell and Gooneratne, 1987). The prehaemolytic

phase is characterised by the release of enzymes from the liver indicative of liver damage, despite plasma Cu concentrations remaining within normal levels (Howell and Gooneratne, 1987; Johnston *et al.*, 2014). The haemolytic phase involves a mass release of Cu by the liver into the bloodstream (Suttle, 2010). The mechanism by which haemolysis occurs is unclear and it is thought that this excess Cu may induce the production of superoxide radicals which cause erythrocyte membrane damage (Howell and Gooneratne, 1987). Clinical signs of the haemolytic phase include dark-coloured urine/blood, dullness, jaundice of the mucous membranes, and death (Perrin *et al.*, 1990). In ruminants, young animals are at a much greater risk of Cu toxicity than their adult counterparts (Todd, 1969). This can be attributed to decreased Cu absorption in adults (<1.0 to 10%) relative to young animals (~70%) caused by the presence of a functional rumen in the mature animal (Underwood and Suttle, 1999). Copper absorption is reduced in mature cattle and sheep by a number of complex reactions in the rumen associated with various antagonists (S and Mo; Allen and Gawthorne, 1987), which are discussed in Section 2.6.

2.7.5 Sub-clinical copper toxicity

There is contradictory evidence regarding the detrimental effects of the prehaemolytic phase on animal performance, with a primary example originating from a dairy farm in Canada where due to a formulation error, cows were exposed to excessive dietary Cu concentrations for an extended period of time (Perrin *et al.,* 1990). Cows which did not succumb to fatality experienced reduced performance in terms of body condition, milk yield and fertility. These cows also experienced reduced health in the form of an increased incidence of metritis and diarrhoea (Perrin *et al.,* 1990). Calves born to cows fed excessive Cu had an increased mortality rate and susceptibility to diseases such as scour, meningitis and naval infections (Perrin *et al.*, 1990). Further evidence that Cu loading has harmful sub-clinical effects is supported by reports of decreased live weight gain (Arthington, 2005), impaired rumen function (Engle and Speers, 2000), and increased calf mortality (Hunter *et al*., 2013).

2.7.6 Excess dietary copper and performance of growing and lactating dairy cattle

Perrin *et al.* (1990) concluded that there was a need to investigate the effects of long-term Cu supplementation on health and performance in cases where excessive Cu loading is not fatal. It should be taken into consideration that the dietary Cu concentration in this case study was estimated at 328 mg Cu/kg fresh in the diet (Perrin *et al.*, 1990). The DM content of the diet was not reported, however at a typical dry matter of 400 g/kg (Sinclair *et al.*,

2017), the Cu content can be estimated at 820 mg Cu/kg DM, 29 times the mean supplementation level of the UK's winter-fed dairy cows reported by Sinclair and Atkins (2015). Engle and Spears (2000) reported a decreased average daily gain (ADG), dry matter intake (DMI), and feed efficiency (Gain/ feed) during the finishing phase when Angus and Crossbred steers were fed a basal diet containing 4.9 mg Cu/kg DM supplemented with 20.0 mg Cu /kg DM as Cu sulfate ($CuSO_4$) to provide a total dietary concentration of 24.9 mg Cu/kg DM (Table 2.6). It was hypothesised that this difference may have been due to an inhibition of rumen function by surplus Cu (Engle and Spears, 2000). Evidence that Cu has the potential to affect rumen function originates from Essig *et al.* (1972), who reported decreased acetic, butyric, propionic and total volatile fatty acids two hours post-feeding when Angus steers were fed a diet with an unknown Cu concentration supplemented with 5.73 g Cu/100 kg of diet (DM not reported). In contrast, Ward and Spears (1997) reported no effect of Cu supplementation level on finishing steer performance when a control diet containing 2.9 mg Cu/kg DM was supplemented with 5 mg/kg DM as $CuSO_4$ to provide a total dietary concentration of 7.9 mg Cu/kg DM.

Table 2.6. Influence of Cu source and inclusion rate on finishing cattle performance.

Cu source	Cu supplementation, mg/kg DM	ADG, kg/d	DMI, kg/d	Gain/ feed	Study length (wks)	Source[1]
$CuSO_4$	0	0.85	9.16	0.09	7	Ward and Spears, (1997)
	5	1.02	9.55	0.01		
$CuSO_4$	0	1.59[a]	9.02[a]	0.18[a]	12	Engle and Spears, (2000)
	20	1.25[b]	8.49[b]	0.15[b]		
	40	1.22[b]	8.59[b]	0.14[b]		
Cu-citrate	20	1.34[b]	8.69[b]	0.16[b]		
Cu-proteinate	20	1.29[b]	8.18[b]	0.16[b]		
Cu-chloride	20	1.31[b]	8.30[b]	0.16[b]		

[a-b] Means within a column of the same study with different superscripts differ ($P < 0.05$). No superscripts within column denote no significant difference between treatments in that study.
[1] Copper concentration of basal diets; Ward and Spears, (1997) = 7.9 mg Cu/kg DM; Engle and Spears (2000), = 4.9 mg Cu/kg DM.

There is also evidence to suggest that over-supplementation during the growing phase has adverse effects on feed intake and efficiency (Gengelbach, 1994). Arthington (2005) reported a lower live weight gain of 0.04 kg/d in crossbred heifers when a control diet containing 7.8 mg Cu/kg DM was supplemented at 120 mg Cu/kg DM as $CuSO_4$ compared

to a gain of 0.22 kg/d for heifers supplemented at 60 mg Cu/kg DM (Table 2.7). Observations of ill thrift have also been identified in Jersey calves exposed to a dietary Cu supply of 35 mg/kg DM in their creep feed (Hunter *et al*., 2013). In contrast, Engle and Spears (2000) reported no effect of Cu source or supplementation rate on Angus and Crossbred steer ADG, DMI, or feed efficiency during the growing phase, when a basal diet containing 10.2 mg Cu/kg DM was supplemented with either 20 mg Cu/kg DM as either $CuSO_4$ or Cu-proteinate. Ward *et al.* (1993) also reported no effect of Cu on ADG or feed intake of growing crossbred steers when a basal diet containing 6.2 mg Cu/kg DM was supplemented with 5 mg Cu/kg DM as either $CuSO_4$ or Cu-lysine. These findings were later reiterated by Ward and Spears (1997) when a basal diet containing 2.9 mg Cu/kg DM was supplemented with 5 mg Cu/kg DM as $CuSO_4$. The inconsistencies surrounding Cu supplementation rate and growing cattle performance may not be a direct effect of dietary Cu supply in isolation but result from a number of complex interactions including; differing levels of dietary antagonists (molybdenum, sulphur and iron), basal rations with differing Cu contents, and varying animal Cu status at the beginning of each study (Engle and Spears, 2000).

Literature is scarce regarding the long-term effects of Cu supplementation on lactating dairy cow performance with few studies extending beyond 16 weeks in duration (Table 2.7). Copper supplementation had no effect on the DMI, milk yield, component content, or somatic cell count (SCC) of Holstein cows when a basal diet containing 8.9 mg Cu/kg DM was supplemented with either 10 or 40 mg/kg DM of $CuSO_4$ to provide a total dietary Cu concentration of either 18.9 or 48.9 mg Cu/kg DM respectively (Engle *et al.*, 2001). These findings support those of other authors who used different Cu sources across various ranges of dietary Cu concentrations (Chase *et al.*, 2000; Sinclair *et al.*, 2013; Table 2.7). Engle *et al.* (2001) did however report an effect of Cu supplementation on milk fatty acid concentration, with higher levels of $C_{12:0}$, and lower levels of $C_{18:2}$ and polyunsaturated fatty acids in the milk of cows supplemented at 40 mg/kg DM relative to those that received no supplemental Cu (Engle *et al.*, 2001). These observations support of those of Morales *et al.* (2000) who reported a decreased biohydrogenation of unsaturated fatty acids in dairy cows subjected to Cu depletion via the addition of dietary Mo and S. It should be taken into consideration that this may either be a direct effect of the altered Cu status and/or the additional S and Mo used to facilitate Cu depletion (Morales *et al.,* 2000). Engle *et al.* (2000) observed a similar effect in steers, reporting lower concentrations of *trans* $C_{18:1}$ in the ruminal fluid of Angus steers when a basal diet containing 5.3 mg Cu/kg DM was supplemented with 20 mg Cu/kg DM as $CuSO_4$ to provide a total dietary concentration of 25.3 mg Cu/kg DM. It was hypothesised that excess Cu exerted this effect by interfering with the reduction

processes during biohydrogenation (Engle *et al.*, 2001). Microorganisms may use enzymes to detoxify excess Cu in the rumen (Wakatsuki, 1995), the presence of which may affect hydrogen transfer (Engle *et al.*, 2001). There is however contrasting evidence to suggest that Cu is involved in the desaturation of $C_{18:0}$ in other tissues such as adipose tissue (Corl *et al.*, 1999). In contrast, Sinclair *et al.* (2013) reported no effect of Cu source on *trans* $C_{18:1}$ and polyunsaturated milk fatty acids when $CuSO_4$ was substituted for Cu-proteinate in the diet.

Table 2.7. Influence of Cu source and supplementation rate on growing cattle performance.

Cu source	Cu supplementation, mg/kg DM	ADG, kg/d	DMI, kg/d	Gain/ feed	Study length (wks)	Source[1]
$CuSO_4$	0	1.1	6.4	0.16	14	Ward *et al.* (1993)
	5	1.1	6.2	0.16		
Cu-lysine	0	1.1	6.4	0.16		
	5	1.0	6.2	0.15		
$CuSO_4$	0	1.02	6.92	0.15	28	Ward and Spears, (1997)
	5	1.03	7.87	0.13		
$CuSO_4$	0	1.55	6.91	0.23	8	Engle and Spears, (2000)
	20	1.50	7.20	0.21		
	40	1.44	6.82	0.21		
Cu-citrate	20	1.48	6.89	0.21		
Cu-proteinate	20	1.52	7.19	0.21		
Cu-chloride	20	1.56	6.91	0.23		
$CuSO_4$	0	$0.14^{a,b}$	5.8	-	12	Arthington, (2005)
	15	0.19^{b}	5.94	-		
	60	0.22^{b}	6.08	-		
	120	0.04^{a}	6.21	-		

[a-b] Means within a column of the same study with different superscripts differ ($P < 0.05$). No superscripts within column denote no significant difference between treatments in that study.
[1] Copper concentration of basal diets; Ward *et al.*, (1993) = 6.2 mg Cu/kg DM; Ward and Spears, (1997), = 2.9 mg Cu/kg DM; Engle and Spears, (2000) = 10.2 mg Cu/kg DM; Arthington, (2005) = 7.8 mg Cu/kg DM.

Table 2.8. Influence of Cu source and inclusion rate on lactating dairy cow performance.

Cu source	Cu supplementation, mg/kg DM	DMI, kg/d	Milk, kg/d	Milk fat, g/kg	Milk protein, g/kg	Study length (wks)	Source[1]
$CuSO_4$	0	26.8	40.0	31	35	9	Engle *et al.*, *(2001)*
	10	26.2	36.7	31	34		
	40	26.7	40.3	30	35		
$CuSO_4$	0	19.0	22.7	35	34	12	Chase *et al.*, *(2000)*
	15	15.9	20.2	42	33		
	30	16.8	16.1	31	32		
Cu-lysine	0	19.0	22.7	35	34		
	15	17.5	24.7	35	31		
	30	18.2	19.9	36	35		
$CuSO_4$	10	22.6	36.1	36	31	16	Sinclair *et al.*, (2013)
Cu-proteinate	10	21.0	34.9	39	31		

[a-b] Means within a column of the same study with different superscripts differ (P < 0.05). No superscripts within a column denote no significant difference between treatments in that study.
[1] Copper concentration of basal diets; Engle *et al.*, (2001) = 8.9 mg Cu/kg DM; Chase *et al.* (2000) = 8 mg Cu/kg DM; Sinclair *et al.* (2013) = 6.4 mg Cu/kg DM.

2.7.7 Excess dietary copper and fertility

Positive effects of Cu supplementation upon fertility in dairy cattle receiving Cu in conjunction with Se and Co have been documented (Black and French, 2004; Mackenzie *et al.*, 2001). Black and French (2004) reported increased conception rates in dairy cows receiving Cu, Se and Co supplementation as either a subcutaneous injection or an intraruminal bolus. This study is however cofounded as Cu was not supplemented in isolation and its individual effects remain unknown (Black and French, 2004). In contrast, Phillippo *et al.* (1987b) reported profound fertility effects on both puberty and conception when Hereford-Friesian heifers were fed a low Cu diet (4 mg /kg DM) with either additional Mo (5 mg/kg DM) or Fe (500 or 800 mg/kg DM) in two experiments. Puberty was observed to occur as normal in the control and Fe supplemented animals but was delayed by 12 and 8 weeks for Mo supplemented heifers in both experiments respectively (Phillippo *et al.*, 1987b). Molybdenum supplementation was blamed for this delay as an equally reduced Cu status was observed in the Fe supplemented animals (Phillippo *et al.*, 1987b). It was hypothesised these effects resulted from a decreasing pulsatile release of luteinising hormone in the affected animals causing anoestrous (McLeod *et al.*, 1982). Supplementation with Mo in the study also reduced conception rates from 69% to 23% in comparison to the control and Fe supplemented groups (Phillippo *et al.*, 1987b), it was

subsequently suggested that Mo supplementation may affect oestrogen metabolism (Phillippo *et al.*, 1987b).

There is little evidence regarding the direct effects of dietary Cu concentration upon fertility in lactating dairy cattle, and that which does exist is conflicting. Jolly *et al.*, (1987) reported no effect on pasture-fed dairy cow fertility in New Zealand when liver Cu concentrations increased from 100 to 350 µmol/kg wet tissue (24 to 84 mg/kg DM; Rosendo and McDowell, 2003) on 2 farms over the course of 10 months. In contrast, Hawkins (2014) reported a decrease in 21-day submission rates from 78 to 75%, in conjunction with a fall in 21-day pregnancy rate from 47 to 43% relative to controls when New Zealand dairy cows received a subcutaneous injection of 200 mg of Cu as Ca Cu EDTA 10 days prior to mating. There was no definitive biological mechanism proposed by which Cu supplementation decreased oestrus demonstration and conception in this case, although Hawkins (2014) did however suggest that the causal factor may be related to oestrus expression. This evidence did lend support to the observations of Cummins and Harris (1984), who reported a decrease in first service conception rate from 75 to 25% relative to controls when Hereford beef heifers received a parental administration of 400 mg of Cu-glycinate 14 days prior to artificial insemination, and led to the conclusion that the parenteral administration of Cu close to mating should be avoided as reproductive performance may be compromised (Cummins and Harris, 1984).

Further evidence that an elevated Cu status may be detrimental to fertility is observed in humans suffering from Wilson's disease (Morimoto *et al.*, 1986). Patients typically experience liver damage at an early age which subsequently leads to menstrual abnormalities (Sass-Kortsak and Bearn, 1978; Cundy *et al.*, 1991). Cundy *et al.* (1991) investigated the cause of amenorrhea in 12 young women with chronic liver disease, they reported reduced levels of follicle stimulating hormone relative to those of healthy test subjects as a result of hypothalamic-pituitary dysfunction. In contrast, the Wilson's disease patients had higher oestradiol levels relative to the control group (Cundy *et al.*, 1991).

Engle *et al.* (2001) suggested that dietary Cu supplementation may influence lactating dairy cow reproductive performance by modulating plasma cholesterol concentrations. Increased serum cholesterol concentrations have been linked with increased fertility (Rodney *et al.*, 2015). Westwood *et al.* (2000) reported an increased conception rate and a shorter calving to conception interval associated with elevated plasma cholesterol. It was hypothesised that

increased cholesterol benefits fertility by enhancing progesterone production (Grummer and Carroll, 1991). The literature is conflicting regarding the effect of Cu status on cholesterol metabolism, several authors have associated increasing Cu supplementation with decreased serum cholesterol concentrations in steers (Engle *et al.*, 2000; Engle and Speers, 2000). In contrast, Engle *et al.* (2001) reported increased serum cholesterol concentrations in dairy cattle when the supplementation of a basal diet containing 5.9 mg Cu/kg DM increased from 10 to 40 mg Cu/kg DM as $CuSO_4$. These differing findings with respect to plasma cholesterol concentrations may have a variety of causes including genetic differences (Hardman *et al.*, 2007), differences between the composition of feedlot and dairy rations (Engle, 2014), and differing levels of mineral antagonists between the various diets (Engle, 2014).

2.7.8 Dietary copper excess and immune function

The effect of Cu deficiency upon the ruminant immune system has been widely discussed (Minatel and Carfignini, 2000), and several studies have related reduced neutrophil phagocytic capacity with decreasing Cu status (Xin *et al.*, 1991; Boyne and Arthur, 1981). Ward *et al.* (1993) reported no effect of Cu source or supplementation level on either the cell-mediated or humoral immune response when a basal diet containing 6.2 mg/kg DM was supplemented with 5 mg Cu/kg DM as either $CuSO_4$ or Cu-lysine respectively. There is however evidence that increasing dietary Cu concentration may modulate certain aspects of the immune system (Goselink and Jongbloed, 2012). Saker *et al.* (1994) reported increased oxidative burst and monocyte phagocytic activity in weaned calves when Cu intake was increased from 43 mg/day to 97 mg/day, approximately equivalent to an increase in dietary Cu concentration from 7 mg/kg DM to 17 mg/kg DM (Hoffman *et al.*, 2008).

There is also evidence linking Cu supplementation levels with milk SCC and clinical mastitis incidence (Goselink and Jongbloed, 2012). Engle *et al.* (2001) reported no effect of $CuSO_4$ supplementation on milk SCC, findings in agreement with those of Chase *et al.* (2000) who also reported no effect of dietary Cu concentration on milk SCC of lactating Holstein dairy cows. It should however be taken into consideration that most mastitis and SCC problems occur around parturition (Dang *et al.*, 2008), whilst both of these studies used mid-lactation dairy cows (Engle *et al.*, 2001; Chase *et al.*, 2000). In contrast, Scaletti *et al.* (2003) reported a lower incidence of mastitis at calving in Holstein cows fed a basal diet containing 6.5 mg Cu/kg DM supplemented with 20 mg Cu/kg DM as $CuSO_4$ to provide a total dietary concentration of 26.5 mg Cu/kg DM. Scaletti *et al.* (2003) also reported lower SCC and

clinical udder scores in Cu supplemented cows after being exposed to an intramammary E.coli challenge. It was hypothesised that this effect may be a result of increased neutrophil function in cows fed the Cu supplemented treatment (Scaletti *et al.*, 2003; Torre *et al.*, 1996).

Evidence relating Cu status and immunity can also be observed in the human population, Turnland *et al.* (2004) reported a decrease in neutrophil numbers coupled with an increase in lymphocyte numbers when men were exposed to excessive levels of Cu supplementation over a period of 129 days. A decrease in soluble interleukin 2 receptors (IL-2R) in the serum of individuals exposed to the high Cu treatment was also reported (Turnland *et al.*, 2004). This soluble receptor has previously been shown to promote lymphocyte proliferation (Lord *et al.*, 2000; Turnland *et al.*, 2004). These findings are consistent with those of Massie *et al.* (1993), who reported decreased IL-2R in mice exposed to high levels of Cu. Turnland *et al.* (2004) concluded that the modulatory effects of high Cu intake upon the immune system are comparable to those observed under situations of Cu deficiency (Percival, 1988; Bonham *et al.*, 2002).

Czlonkowska and Milewski (1976) reported an increased humoral immune response in patients suffering from Wilson's disease as exemplified by an increased antibody titre against *Enterobacteriaceae common antigen* (Kunin's CA antigen). Depressed cell-mediated immunity in the form of a lower response to an *E.coli* skin test was also observed (Czlonkowska and Milewski, 1976). Czlonkowska and Milewski (1976) concluded these observations were a result of increased free Cu in the serum during the haemolytic phase leading to a suppression effect on the T cells and an overactivation of B cells (Czlonkowska and Milewski, 1976; Walshe and Cummings, 1961). In contrast, Turnland *et al.* (2004) identified a significantly decreased humoral response as evidenced by a lower titre of antibodies against the influenza vaccine (Beijing strain) in healthy humans receiving high dietary Cu concentrations. The inconsistency in findings between these sources may either be a result of a much higher Cu status of the patients suffering from Wilson's disease (Czlonkowska and Milewski, 1976), or a result of different immune adjuvants used in each study causing different responses(Dalsgaard *et al.*, 1990), although neither study declared the immunological adjuvants used (Czlonkowska and Milewski; Turnland *et al.*, 2004).

2.8 Determining copper status

2.8.1 Plasma copper and ceruloplasmin

The measurement of total plasma Cu as an indicator of Cu status is thought to offer little advantage over Cp activity with both parameters shown to be highly correlated (Legleiter and Spears, 2007), with estimations of approximately 80-95% of plasma Cu being present as ceruloplasmin (Suttle 2010; Terada *et al.*, 1995). Normal plasma Cu concentrations are thought to range from 9 to 19 µmol/L in both cattle and sheep (Suttle, 1994; Laven and Livesey, 2004; 2005), with Cu-responsive disorders being observed at values ranging from 3 to 9 µmol/L (Underwood and Suttle, 1999), and plasma Cu concentrations as high as 181 µmol/L reported in clinically toxic cattle (Bidewell *et al.*, 2012). Evidence suggests three possible relationships between plasma Cu and Cu status including; no relationship between the two parameters (Sinclair *et al.*, 2017; Table 2.9), a decrease in plasma Cu associated with decreasing Cu status in cases of Mo supplementation (Hussein, 2017; Humphries *et al.*, 1983), or an increase in plasma Cu associated with decreasing Cu status and Mo supplementation (Du Plessis *et al.*, 1999). Potential hypotheses for these inconsistent effects have been directed towards differing thiomolybdate speciation and/or absorption between studies (Williams, 2004). Dias *et al.* (2013) conducted a meta-analysis relating plasma Cu to dietary concentrations of Cu, S and Mo, and concluded that any prediction equation would be of limited use, and it was only in cases of excessively high or low hepatic Cu that plasma Cu concentration could be used as a reliable indicator of Cu status (Laven and Livesey, 2005; Dias *et al.*, 2013).

Ceruloplasmin is a Cu containing metalloenzyme found in the plasma of most vertebrate species (Hellman and Gitlin, 2002), although the liver acts as the primary source of serum Cp, extrahepatic Cp gene expression has been observed in the brain, lungs and testes (Hellman and Gitlin, 2002; Fleming and Gitlin, 1990; Klomp *et al.*, 1996). Serum Cp is found in two forms in healthy animals, approximately 90% of circulating Cp is present as holoceruloplasmin and 10% is present as apoceruloplasmin (Matsuda *et al.*, 1974). Apoceruloplasmin is formed when Cu fails to incorporate into the enzyme during formation (Hellman and Gitlin, 202), it is devoid of ferroxidase activity and is rapidly catabolised with a half-life of approximately 5 hours (Matsuda *et al.*, 1974). In contrast, it is estimated that holoceruloplasmin has a half-life of approximately 5.5 days (Gitlin and Janeway, 1960). During Cu deficiency the proportion of total Cp present as apoceruloplasmin within the bloodstream has been shown to increase (Holtsman and Gaumnitz, 1970; Gitlin *et al.*, 1992). Ceruloplasmin has a variety of functions within the animal (Hellman and Gitlin, 2002), it not only acts as a transport protein by delivering Cu to the cell membrane receptor

(Saenko *et al.*, 1994), but also an acute phase protein during the inflammatory response in incidences of trauma and/or infection (Matsuda *et al.*, 1974). The enzyme also functions as a membrane ferroxidase and is responsible for the extracellular oxidisation of Fe for subsequent cellular uptake (Stearman *et al.*, 1996).

Ceruloplasmin activity has a normal range of 12-24 mg/dL in serum (Williams, 2004), and has been used previously as an indicator of Cu status in ruminants (Mackenzie *et al.*, 1997b). Its activity has been shown to decrease during cases of dietary Mo exposure or Cu deficiency (Humphries *et al.*, 1983; Blakey and Hamilton, 1985). The precise mechanism by which this decrease in activity occurs is however unclear as described in Section 2.5.2. Ceruloplasmin activity does not consistently reflect changes in Cu status in cattle and sheep (Sinclair *et al.*, 2013; Hussein, 2017; Table 2.9). The enzyme's role as a minor acute-phase protein may be one potential reason for this (Kaya *et al.*, 2016), as there have been cases where vaccines and infections have been shown to induce Cp synthesis in hypocupraemic animals (Suttle, 1994). Given that Cp is produced by the liver at a constant rate provided hepatic Cu storage is not limiting (Hellman and Gitlin, 2002), Cp activity may only be useful as an indicator in cases of Cp inhibition by higher systemic thiomolybdate concentrations (Suttle, 1991), and/or Cu responsive disorders due to a lack of hepatic Cu to sufficiently synthesise holoceruloplasmin (Suttle, 2010).

2.8.2 Ceruloplasmin to plasma copper ratio

The ceruloplasmin to plasma Cu ratio (Cp: plasma Cu) has been advocated as a better indicator of functional Cu status within the animal than either parameter in isolation (Mackenzie *et al.*, 1997b). The ratio serves to relate total plasma Cu to Cp activity, with a decreasing ratio value indicating a greater presence of TMs within the animal (Telfer *et al.*, 2004). Mackenzie *et al.* (1997b) proposed that a theoretical Cp: plasma Cu ratio of 2:1 indicated no absorption of TMs into the blood, a value less than 2.0 indicated partial absorption of TMs and the initialisation of Cu-dependent enzyme inhibition, a value less than 1.5 indicated TM problems, and values less than 1.0 indicated a severe TM problem (Mackenzie *et al.*, 1997b).

The validity of this ratio was to some extent confirmed by Mackenzie *et al.* (2001) who reported improved fertility rates as a result of the Cu supplementation of animals with low Cp: plasma Cu ratios. Telfer *et al.* (2004) also reported Cu-thiomolybdate absorption in dairy cattle with decreased Cp: plasma Cu ratios. There is however evidence from other studies

calling into question this hypothetical 2.0 value, where animals have been in positive hepatic Cu balance when fed diets containing low dietary S and Mo concentrations despite ratios well below the 2.0 considered to signify TM absorption (Hussein, 2017; Sinclair *et al.*, 2013; Sinclair *et al.*, 2017; Table 2.9). Indeed, studies have identified poor correlations between this ratio and hepatic Cu concentration (r^2 = 0.2444; Williams, 2004), in addition to the lack of a decreasing ratio in cases where elevated dietary Fe has reduced hepatic Cu (Williams, 2004). The apparent inability of dietary Fe to affect the Cp: plasma Cu ratio may in part be due to contrasting mechanisms of antagonism between TMs and Fe as discussed in Section 2.6. The use of this ratio has become a much-debated topic and may explain a lack of widespread use in full journal articles (Suttle, 2016).

One of the major criticisms of this ratio is that Cp activity loss is variable during serum clotting and can be as high as 20% (Suttle, 2002; Laven *et al.*, 2011). Telfer *et al.* (2004) countered this criticism by suggesting that the marginal category of 1.5 to 2.0 acts as a grey area accounting for this loss and/or possible molybdenum toxicity. Either way perhaps the most significant issue with the use of this ratio is the range of enzyme assays used to determine Cp activity across the globe, their instability, and a lack of standardisation between research laboratories (Laven *et al.*, 2011), with the result that comparison between treatments within a study may be more meaningful than absolute ratio values (Laven *et al.*, 2011).

2.8.3 Superoxide dismutase

Superoxide dismutase is a Cu-dependent enzyme whose antioxidant action is vital for aerobic cell survival (Fridovich, 1975). The enzyme is responsible for the detoxification of superoxides by conversion to oxygen and hydrogen peroxide as shown below (Lippard and Berg, 1994):

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

Superoxide radicals are formed during normal metabolic processes such as respiration and phagocytosis (Fukai and Ushio-Fukai, 2011). Failure of these free radicals to be removed from the system results in oxidative stress (Araya et al., 2006; Davis, 2003). This is a state where free radicals induce effects such as cell-membrane and DNA damage (Osredkar and Sustar, 2011). Superoxide dismutase is found within mammals as Cu/Zn superoxide

dismutase (SOD_1), and Mn superoxide dismutase (SOD_2) (Cao et al., 2008; Borgstahl et al., 1996). Chang et al. (1988) identified the majority of SOD_1 to be found within the cystoplasmic matrix (73.1%), and nucleus (11.9%) of the cell. In contrast, SOD_2 resides predominantly within the mitochondria of the cell (Slot et al., 1986). Superoxide dismutase 3 is 60% homologous to SOD_1 including the Cu and Zn per subunit (Gao *et al.*, 2008), it is however extracellular, highly tissue specific, and found primarily in the lungs, heart, blood vessels, and kidneys (van der Vliet, 2015). Tests for whole blood SOD fail to distinguish between these forms of the enzyme (Fridovich, 1975), however as SOD_1 represents the predominant form in the bloodstream, total SOD levels may have some potential to reflect copper status (Williams, 2004; Suttle and McMurray, 1983).

Superoxide dismutase activity has been suggested as a long-term indicator of Cu status in comparison to Cp or plasma Cu (Suttle and McMurray, 1983). This is due to the relatively long lifespan of erythrocytes (92 days) in comparison to Cp (5.5 days), which results in a gradual decrease in whole-blood SOD activity in response to a decreasing Cu status within the animal (Ward and Spears, 1997; Suttle and McMurray, 1983). Superoxide dismutase activity therefore has been hypothesised to be a much better indicator of long-term Cu deficiency as it may be less sensitive to short-term changes in dietary Cu concentration (Paynter, 1987). Superoxide dismutase is predominantly contained within the membrane bound erythrocyte whereas Cp is free within the plasma of the animal (Slot *et al.*, 1986; Suttle, 2010). Little is known about the ability of thiomolybdates to travel across this membrane and sequester Cu from SOD, however an inability of the complex to do so may explain cases where there is inhibition of Cp but not SOD when sheep and dairy cows were fed diets with additional S and Mo (Hussein, 2017; Sinclair *et al.*, 2017). Ogra *et al.* (1996) reported that TM_4 did remove Cu from the SOD of rats *in vitro* if Cu bound metallothionein was present.

Table 2.9. Effects of basal forage type or Cu source on indicators of Cu status used in cattle and sheep studies.

Cu source/ Predominant basal forage type[1]	-/+[2]	Pl-Cu, µmol/L	Cp, mg/dL	Cp, Pl-Cu	SOD, U/g of Hb	Hepatic Cu Δ, mg/kg DM/d	Source
MS	-	13.3	17.9[a]	1.37	2960	0.66[a]	Sinclair *et al.*, (2017)
MS	+	13.7	15.9[c]	1.22	2841	0.11[b]	
GS	-	14.3	20.3[b]	1.41	2954	0.84[a]	
GS	+	13.7	18.1[c]	1.36	2915	-0.62[c]	
$CuSO_4$	-	13.5	20.2	1.53	2670	0.33[a]	Sinclair *et al.*, (2013)
$CuSO_4$	+	12.9	18.8	1.49	2511	-0.91[b]	
Cu-proteinate	-	13.2	18.9	1.43	2532	-0.07[a]	
Cu-proteinate	+	12.8	19.8	1.53	2557	-0.87[b]	
GH	-	15.1[a]	10.9[a]	0.73[a]	1836	-0.06[a]	Hussein, (2017)
GH	+	13.5[b]	8.7[b]	0.66[b]	1891	-0.18[c]	
MS	-	15.1[a]	10.6[a]	0.70[a]	1880	0.13[b]	
MS	+	12.8[b]	9.1[b]	0.68[b]	1763	-0.10[c]	
DGP	-	16.5	12.0	0.74	2384	0.50[a]	Hussein, (2017)
DGP	+	16.5	11.7	0.74	2463	0.20[b]	
MS	-	16.5	12.4	0.75	2529	0.19[a]	
MS	+	16.6	11.9	0.73	2428	-0.07[b]	

[a-c] Means within a column of the same study with different superscripts differ ($P < 0.05$).
[1] MS = maize silage, GS = grass silage, GH = grass haylage, DGP = dried grass pellets.
[2] Control (-) = no additional S and Mo, antagonist (+) = additional S and Mo.

2.8.4 Hepatic copper

The liver is generally considered the principal Cu storage organ within the body (Laven and Livesey, 2005), and under incidences of Cu deprivation or over-supply, one of the first biochemical changes to occur is a decrease or increase in hepatic Cu concentration (Suttle, 2010). Copper distribution within the hepatocyte was discussed at length in Section 2.4. There is however debate surrounding the liver Cu concentration thresholds at which clinical symptoms of either under or over-supplementation may occur as these can be influenced by species (Grace and Wilson, 2002), breed (Legleiter and Spears, 2007; Phillippo *et al.*, 1987a), and level of performance (Du *et al.*, 1996). For example, Holstein cows are generally reported to have a greater ability to either withstand low or high liver Cu concentrations whilst sustaining normal Cp synthesis relative to Jersey cows (Du *et al.*,

1996). Current thresholds for normal liver Cu concentrations range from 19 mg Cu/kg DM to 508 mg Cu/kg DM for cattle (Laven and Livesey, 2005; Livesey *et al.*, 2002), although it has been reported that liver Cu values ranging from 6.5 to 19.5 mg Cu/kg DM may pose a possible risk, and values of < 6.5 mg Cu/kg DM a high risk of bovines developing Cu responsive disorders (Suttle, 2010).

Several authors have noted an uneven distribution of Cu within the liver (Puls, 1994; Braselton *et al.*, 1997; Miranda *et al,* 2010). Miranda *et al.* (2010) reported the highest hepatic Cu concentrations in the left lobe, and lowest concentrations in the caudate and quadrate lobes of bovine liver respectively (Figure 2.4). Varying distribution of Cu within the liver is hypothesised to result from a variety of factors including genetics (Underwood and Suttle, 1999), sex (Miranda *et al.*, 2006), and age (Miranda *et al.*, 2006). Copper has been shown to concentrate in the centrilobular zones of sheep in cases of dietary deprivation (Kumaratilake and Howell, 1987), with a subsequent spread to the extremities of the liver as total hepatic Cu content increases during repletion (Kumaratilake and Howell, 1987). This pattern of accumulation may however differ in adult cattle where there are greater differences in the distance between the central blood vessels and superficial hepatic lobes (Miranda *et al.*, 2010). It is hypothesised that overall differences in Cu content between liver lobes result from differences in blood supply to those lobes and their bile excretion capacity (Miranda *et al.*, 2010). Miranda *et al.* (2010) did however conclude that sampling a section of the external right lobe of the liver with a needle is an adequate reflection of hepatic Cu status in cattle.

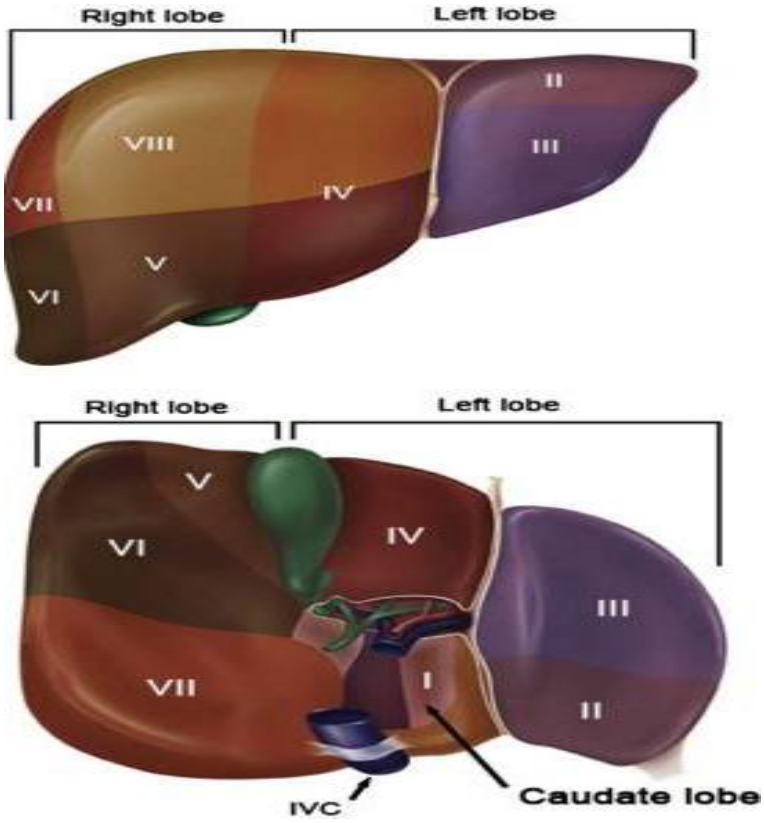

Figure 2.4. Lobe distribution in the liver (Abdel-Misih and Bloomston, 2014).
I = caudate lobe, II = Couinard's left lateral segment II, III = Couinard's left lateral segment III, IV = left medial segment, V = right anterior lobe V, VI = right posterior lobe VI, VII = right posterior lobe VII, VIII = right anterior lobe VIII, IVC = inferior vena cava. External right lobe = area sampled during liver biopsy in cattle (Miranda *et al.*, 2010).

2.8.5 Other indicators of copper status

There are other potential indicators of Cu status in cattle, these predominantly make use of the element's role as a co-factor in over 300 Cu-dependent enzymes (Bonham *et al.*, 2002; Linder, 1991 Prohaska, 2006) but are not widely utilised and/or proven in literature (Paynter, 1987). Lysyl oxidase is a Cu-dependent enzyme responsible for the cross-linkage of polypeptide chains including collagen and elastin (Linder, 1991; Rucker *et al.*, 1996), and as such the enzyme is found in high concentrations within the heart, skin and cartilage cells (Rucker *et al.*, 1996; 1998). During Cu deficiency, a reduction in lysyl oxidase activity may cause defects relating to elastic tissue formation as exemplified by pulmonary disease and spinal curvature (O'Dell, 1976). The suitability of this enzyme as an indicator of Cu status in ruminants however remains unassessed (Suttle, 2010). Tyrosinase is another Cu-dependent enzyme responsible for catalysing the formation of the black pigment melanin from tyrosine (Davis and Mertz, 1987). Decreased production of this enzyme during instances of Cu deficiency results in a greying of black or brown hair in both cattle and sheep (Underwood, 1977). The behaviour of tyrosinase during cases of decreasing Cu

status appears similar to that of ceruloplasmin, where a change in coat colour was reported in cattle receiving supplemental dietary Mo but no change was reported in those receiving supplemental Fe despite a comparative decrease in Cu status (Phillippo *et al.*, 1987a, b). These findings may have been a result of TM absorption in the Mo supplemented diets (Williams, 2004).

Amine oxidase (AO) is another Cu-dependent enzyme involved in the cross-linkage of collagen and elastin (Bachrach, 1985). The enzyme's plasma concentration has previously been reported to be relatively resistant to change when cattle become Cu deficient (Mills *et al.*, 1976). Studies have since reported reduced AO activity in Jersey steers following the intravenous administration of TM_2 and TM_3 (Mulryan and Mason, 1986), as well as an inhibition of the enzyme *in vitro* by TM_2 (Mulryan and Mason, 1987). Legleiter and Spears (2007) identified that the enzyme was highly correlated to liver Cu concentration, plasma Cu level, and Cp activity (Pearson R = 0.73 to 0.87) when Cu deficiency was experimentally induced in Angus steers through the dietary inclusion of Mn (500 mg/kg DM in diet 1 vs. 20 mg/kg DM in diet 2) and Mo (2 mg/kg DM in diets 1 and 2) respectively. These enzymes however remain relatively unused indicators of Cu status for a number of reasons such as their biological significance regarding Cu metabolism in ruminants is not well understood (Bachrach, 1985), Cp is already used as a potential indicator of TM absorption (Suttle, 1991), and they have not been widely validated in literature (Legleiter and Spears, 2007).

Enzymes relating to liver function represent other possible indicators of Cu status (Bidewell *et al.* 2012). These indicators utilise the process of liver degeneration at elevated liver Cu concentrations prior to and during the onset of hepatic necrosis (Giannini *et al.*, 2005). Where hepatocytes rupture at these elevated Cu concentrations releasing their constituents into the bloodstream resulting in elevated plasma aspartate aminotransferase (AST), Ɣ-glutamyltransferase (GGT), glutamate dehydrogenase (GLDH), and bilirubin (Bidewell *et al.*, 2012; Humann-Ziehank *et al.*, 2001). The main criticism of these enzymes is that they do not give an indication of changing Cu status when hepatic Cu concentrations are within the normal range (Johnston *et al.*, 2014), hence their lack of use in studies relating to Cu metabolism (Engle 2014, Suttle, 2010). They have however proven useful as indicators of herd Cu status in veterinary case studies investigating clinical cases of Cu toxicity as a result of suspected over-supplementation (Bidewell *et al.*, 2012; Johnston *et al.*, 2014).

2.9 Conclusion

Cows within the UK dairy sector are currently being over-supplied with dietary Cu which may have implications for food safety with liver containing high concentrations of the element entering the supply chain. Following an extensive review of the literature, there is little evidence regarding the long-term effects of Cu over-supplementation on the performance, health and fertility of growing and lactating dairy cattle. It is also apparent that a poor understanding of factors affecting Cu absorption may be contributing to this over-supplementation.

2.10 Knowledge gap

Studies to date reporting the effects of dietary Cu concentration on bovines have tended to focus on performance, been short-term in duration, and fixated on beef cattle (Engle and Spears, 2000; Engle, 2014). There is however anecdotal evidence from practising vets to suggest negative health effects of elevated hepatic Cu concentrations (Howie, 2017), and reports of decreased performance in lactating dairy cows in cases of accidental over-supplementation (Perrin *et al.*, 1990). One of the hypothesised reasons for this over-supplementation of Cu is a lack of understanding regarding the interactions between Cu, S and Mo in the rumen (Kendall *et al.*, 2015). Equations used to predict Cu requirements as a result of S and Mo antagonism were developed in the 1970's using semi-purified diets in sheep (Suttle and McLauchlin, 1976; Suttle, 2016), they do not however take account of other dietary factors (Suttle and McLauchlin, 1976), and have since been charged with antiquation by some (Suttle, 2016). There is evidence that other dietary factors may influence Cu metabolism with a variety of studies identifying varied Cu absorption when one forage type is substituted for another (Hussein, 2017; Sinclair *et al.*, 2017; Table 2.9), although reasons for these differences have thus far remained unclear.

The objectives of this thesis are:

- To determine the effects of Cu supplementation level during the rearing phase on replacement Holstein-Friesian dairy heifer performance, health, fertility and Cu status.
- To determine the effects of lifetime Cu supplementation level on replacement Holstein-Friesian performance, health and fertility during the first lactation.

- To determine mechanisms by which basal forage type effects Cu absorption efficiency in lactating Holstein-Friesian dairy cows.
- To evaluate methods of measuring Cu status.
- To provide recommended Cu supplementation strategies to nutritionists and dairy farmers in order to optimise cow performance and health.

CHAPTER 3: Materials and Methods

This Chapter contains methods which are common across all three subsequent experimental Chapters 4 to 6, any methods which are specific to a particular experimental section have be outlined in detail in that particular Chapter.

3.1 TMR, concentrate, and grass sample analysis

Feed samples collected across all three studies were analysed according to AOAC (2012) for dry matter (DM; 934.01), crude protein (CP; 990.03), ether extract (EE; 2003.5), and ash (942.05) at Harper Adams University. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) content was analysed according to Van Soest *et al.* (1991), and the starch content was analysed according to ISO 6493 (2000). Prior to all laboratory analysis all dried feed samples were milled using a cyclone mill (Cyclotec, Warrington, UK) with a 1 mm sieve.

3.1.1 Crude protein content

The CP content of the TMR, concentrate, and grass samples was calculated from total feed nitrogen (N) content which was determined using the Dumas method according to AOAC (2012; 988.05) conducted via a LECO FP 528 (LECO Corp, Stockport, UK). The CP content of the feed was calculated using the equation:

$$CP\ (g/kg\ DM) = N\ x\ 6.25\ \times 10 \qquad \text{Equation 3.1}$$

3.1.2 Organic matter (OM) and ash

The ash content of the feed samples was determined according to AOAC (2012), approximately 2 g (1.8 to 2.2 g) of the sample was placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) at a temperature of 550 ºC for 4 hours.

3.1.3 Neutral detergent fibre

The NDF content of the TMR, concentrate and grass samples was determined using a Fibertec system (1020 Hot Extractor, FOSS, Warrington, UK) using the procedure as described by Van Soest *et al.* (1991). Both sodium sulphite and heat stable α-amylase were

used during NDF determination and resulting NDF values were expressed exclusive of residual ash. Neutral detergent fibre reagent was prepared as follows; 93 g of di-sodium ethylene diamine tetra-acetic acid dehydrate (EDTA), 34 g of sodium tetraborate ($Na_2B_4O_7.10H_2O$), 150 g of sodium dodecyl sulphate (SDS), 22.8 g of anhydrous sodium hydrogen phosphate (Na_2HPO_4), and 50 ml of tri-ethylene glycol were dissolved in approximately 3 L of hot distilled water. The resulting solution was allowed to cool to room temperature and made up to 5 L using distilled water (room temperature). The pH of the reagent was adjusted to lie within the range of pH 6.9 to 7.1 via the addition of 0.1 M hydrochloric acid (HCL) or 0.1 M of sodium hydroxide (0.1 M NaOH). Alpha amylase solution was subsequently prepared as follows; 2.8 g of α-amylase from *Bacillus subtilis spp* (80 EU/mg, Sigma, Gillingham, UK) was in distilled water. The resulting solution was then made up to 100 ml by the addition of 10 ml of tri-ethylene glycol.

Approximately 0.5 g (0.4 to 0.6 g; exact weight recorded to 4 decimal places) of the dried and milled sample was weighed into a pre-weighed glass crucible (porosity 1, Soham Scientific, Ely, UK). Each crucible was then placed in the Fibertec apparatus (1020 Hot Extractor, FOSS, Warrington, UK) and 25 ml of NDF reagent followed by 5 drops of octanol (reagent grade, Sigma Aldrich, Dorset, UK) were added. The NDF reagent containing the samples was then brought to boiling temperature and the samples were digested for 30 minutes. Following initial digestion, another 25 ml of NDF reagent was added to the crucibles followed by 0.5 g of sodium sulphite and 2 ml of α-amylase. The samples were then brought up to boiling temperature for a second time, and allowed to digest for a further 30 minutes. The heat was subsequently turned off and the NDF reagent removed by filtration. The sample was then washed and filtered 3 times with 25 ml of hot distilled water (approximately 80 ºC). Once all of the NDF reagent had been removed by washing, a further 25 ml of hot distilled water was added to the sample followed by 2 ml of α-amylase. The samples were allowed to stand in this solution for 15 minutes, and subsequently filtered and washed a further 3 times using hot distilled water. Crucibles were then removed from the Fibertech apparatus and placed in an oven at 105 ºC to dry overnight. The crucibles were then removed from the oven and allowed to cool to room temperature in a desiccator. Once cooled, crucibles were re-weighed to give a dry weight. The crucibles containing the samples were then placed in a muffle furnace at 550 ºC for 4 hours, after which the samples were again allowed to cool to room temperature in a desiccator. Once cooled, the crucibles were re-weighed. The NDF content of the feed samples was calculated using the following equation:

$$NDF\ (g/kg\ DM)\ = \frac{a-b}{c} \times 100\ \times \frac{1000}{d} \qquad \text{Equation 3.2}$$

Where:

a = crucible mass + dried sample (g)
b = crucible mass + ashed sample (g)
c = initial sample mass added to crucible (g)
d = sample dry matter (g)

3.1.4 Acid detergent fibre

The ADF content of the TMR, concentrate and grass samples was determined according to Van Soest *et al.* (1991) using a Fibertec system (1020 Hot Extractor, FOSS, Warrington, UK), and expressed exclusive of residual ash. The reagent used for ADF analyses was prepared by adding 20 g of cetyltrimethylammonium bromide (CETAB; Sigma Aldrich, Dorset, UK) to 1 L of 1 M sulfuric acid (Sigma Aldrich, Dorset, UK).

Approximately 0.5 g (0.4 to 0.6 g; exact weight recorded to 4 decimal places) of the dried and milled sample was weighed into a pre-weighed glass crucible (porosity 2, Soham Scientific, Ely, UK). Each crucible was then placed in the Fibertec apparatus (1020 Hot Extractor, FOSS, Warrington, UK) and 100 ml of ADF reagent added. The samples were subsequently brought to boiling temperatures and digested for 60 minutes. Once digestion was complete, samples were filtered and washed 3 times using 25 ml of hot distilled water (approximately 80 ºC). Crucibles were then removed from the Fibertech apparatus and placed in an oven at 105 ºC to dry overnight. Samples were allowed to cool to room temperature, reweighed and placed in a muffle furnace set at 550 ºC for 4 hours. The crucible containing the ashed sample was subsequently allowed to cool again in a desiccator and reweighed. The ADF content of the feed samples was calculated using the following equation:

$$ADF\ (g/kg\ DM) = \frac{a-b}{c} \times 100 \times \frac{1000}{d} \qquad \text{Equation 3.3}$$

Where:

a = crucible mass + dried sample (g)
b = crucible mass + ashed sample (g)
c = initial sample mass added to crucible (g)
d = sample dry matter (g)

3.1.5 Ether extract

The EE content of the TMR, concentrate, and grass samples was determined using a Soxtec apparatus (FOSS, Warrington, UK) according to AOAC (2012). Approximately 1.0 g (0.9 to 1.1 g; exact mass recorded to 4 decimal places) of previously dried and milled sample was accurately weighed into a cellulose extraction thimble (Whatman Plc, Maidstone, UK) which was then plugged with cotton wool. The thimbles and pre-weighed extraction cups were then placed in the Soxtec apparatus (FOSS, Warrington, UK). The samples were subsequently boiled in 25 ml of petroleum ether (Fisher Scientific, UK) for 1 hour, raised, and rinsed in the evaporated solvent for an additional 15 minutes. The petroleum ether was then syphoned off in the apparatus, the extraction cups were removed, and allowed to cool. Once the extraction cups had reached room temperature, they were reweighed and feed EE content was determined using the following equation:

$$Ether\ extract\ (g/kg\ DM) = \frac{a-b}{b} \times 1000 \times \frac{1000}{d} \qquad \text{Equation 3.4}$$

Where:

a = extraction cup mass + ether extract (g)
b = extraction cup mass (g)
c = initial sample mass (g)
d = sample dry matter (g)

3.1.6 Starch

The starch content of the TMR and concentrate samples was determined at Sciantec Analytical (Stockbridge Technology Centre, North Yorkshire, UK) by the polarimetric method as described by ISO 6493 (2000).

3.1.7 Water-soluble carbohydrate

The water-soluble carbohydrate (WSC) content of the TMR, concentrate, and grass samples was determined according to Thomas (1977). This test operates on the principle that carbohydrate forms a blue-green complex when heated in anthrone reagent (MAFF, 1986), the degree of colour change can then be compared to a standard curve generated from analysing known concentrations of glucose using the same technique (MAFF, 1986). The anthrone reagent was prepared by adding 380 ml of concentrated sulfuric acid (Fischer Scientific, Loughborough, UK), 0.5 g of thiourea (Sigma-Aldrich, Dorset, UK), and 0.5 g of anthrone (Sigma-Aldrich, Dorset, UK) to 165 ml of distilled water. The stock standard solution was prepared by dissolving 0.4 g of D-glucose (Fischer Scientific, Loughborough, UK) in 500 ml of distilled water. Once prepared, the stock standard solution was diluted with

distilled water to produce 5 working standards with glucose concentrations of 0.00, 0.04, 0.08, 0.16, 0.20 mg/ml (equivalent to 0, 40, 80, 160, and 200 g WSC/kg DM). The samples were then extracted as follows; 0.2 g (0.19 to 0.21 g; exact weight recorded to 4 decimal places) of dried ground sample was accurately weighed into a 250 ml shaking bottle, 200 ml of distilled water was added, samples were placed on an HS 501 horizontal shaking machine (IKA England Ltd, Oxford, UK) for 1 hour and filtered through a Whatman No 1 (GE Healthcare, Massachusetts, US) filter paper.

The working standards and sample extracts were analysed as follows; 2 ml of each working standard and sample extract were pipetted into 50 ml glass culture tubes (DWK Life Sciences, Hattenberstr, Germany) and allowed to cool in water containing ice for 10 minutes. Once cooled, 10 ml of anthrone reagent was added down the side of the tube so that it formed a separate layer beneath the sample. The tubes were then loosely stoppered, vortex mixed using an MT-20 vortex mixer (Philip Harris Ltd, Shenstone, UK) and placed in a boiling water bath (S. and J. Jupiter and Company, Essex, UK) for 20 minutes. The tubes were then removed from the water bath and allowed to cool in water containing ice, and the absorbance of each solution was then measured using a Jenway 6305 spectrophotometer (Jenway, Staffordshire, UK) at a wavelength of 620 nm. The readings from the working standards were then used to construct a standard graph of absorbance values (x-axis) against WSC concentration (y-axis). The following equation was generated from the graph and used to calculate the WSC content of the samples:

$$WSC\ content\ (g/kg\ DM) = mx \qquad \text{Equation 3.5}$$

Where:

m = the gradient of the line

x = the absorbance of the sample

3.1.8 Feed minerals

The TMR, concentrate and grass samples were analysed for Ca, P, Mg, S, Cu, Mo, Fe, Zn, and Mn content according to the procedure described by Cope *et al.* (2009). Approximately 0.5 g (0.48 to 0.52g; exact weight recorded to 4 decimal places) of previously dried and milled feed sample was accurately weighed into a 50 ml DigiPREP tube (QMX Laboratories Ltd, Essex, UK), to which 1 ml of concentrated trace element grade HCL (Fischer Scientific, Loughborough, UK) and 6 ml of concentrated trace element grade nitric acid (HNO_3; Fischer Scientific, Loughborough, UK) was added. The DigiPREP tubes were then placed in a DigiPREP heating block (QMX Laboratories Ltd, Essex UK). The temperature of the

samples was altered in a step-wise fashion as follows; increased to 45 ºC over a period of 30 minutes, held at 45 ºC for 1 minute, increased to 65 ºC over a period of 25 minutes, held at 65 ºC for 5 minutes, increased to 100 ºC over a period of 15 minutes, and finally refluxed using watch glasses at 100 ºC for 40 minutes for the concentrates, and 45 minutes for the TMR and grass samples. Samples were then allowed to cool to room temperature and made up to 50 ml with type-1 water.

The samples were then diluted 1:20 using an acidic diluent consisting of 0.50 % trace element grade HNO_3 (Fischer Scientific, Loughborough, UK), 2.00 % high performance liquid chromatography grade (HPLC) methanol (Sigma-Aldrich, Dorset, UK) and 0.05 % Triton X-100 (Fischer Scientific, Loughborough, UK) in type-1 water. Blank and calibration standards were prepared containing 0, 10, 50, 100, 200, and 400 μg/kg of the minor elements Cu, Mo, Fe and Zn (TraceCert, Sigma-Aldrich, Dorset, UK), and 0, 50, 250, 500, 1000, and 2000 μg/kg of the major elements Ca, P, Mg and S in a solution of 0.50 % concentrated HNO_3, 2.00 % HPLC grade methanol, and 0.05 % Triton-X 100. Diluted feed samples were subsequently analysed for the aforementioned elements by inductively coupled plasma mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield UK). The certified EU reference samples BCR-129 (Hay; Sigma-Aldrich, Dorset, UK) and BCR-708 (Dairy feed; Sigma-Aldrich, Dorset, UK) were routinely extracted and analysed in order to ensure precision and accuracy (Table 3.1).

Table 3.1. Certified feed reference material results of ICP-MS analyses.

	Certified reference material[1]					
	EU BCR-129, hay			EU BCR-708, dairy concentrate		
Element	Certified concentration	Analysed concentration	%[2]	Certified concentration	Analysed concentration	%[2]
	g/kg DM			g/kg DM		
Ca	6.40 ± 0.10	6.44 ± 0.07	101	4.8 ± 0.5	4.9 ± 0.2	102
P	2.36 ± 0.07	2.34 ± 0.04	99			
Mg	1.45 ± 0.04	1.26 ± 0.02	87	1.47 ± 0.22	1.57 ± 0.5	107
S	3.16 ± 0.04	2.84 ± 0.05	90			
	mg/kg DM			mg/kg DM		
Cu	10	10.8 ± 0.8	108	37 ± 4	39 ± 1	105
Mo	1.0	1.0 ± 0.1	100			
Zn	32.1 ± 1.7	30.7 ± 1.2	96			
Fe	114	106.5 ± 2.5	93			
Mn	72	73.0 ± 3.8	101			

[1] Uncertainties expressed as the half-width of the 95% confidence interval of the mean.
[2] Percentage recovered = %.

3.2 Blood sample collection, storage and analysis

3.2.1 Blood sample collection

Blood samples were collected from cows and heifers at 11:00h by jugular venepuncture using an 18G x 1.5" needle (Becton Dickinson Vacutainer Systems, Plymouth, UK) into vacutainers (BD Vacutainer, Plymouth, UK) containing the following anti-coagulants; lithium heparin, fluoride/oxalate, dipotassium ethylenediaminetetraacetic acid (K_2EDTA), tripotassium EDTA (K_3EDTA), and silica gel. Lithium heparin, fluoride/oxalate, and K_3EDTA vacutainers were immediately centrifuged (Beckman Avanti 30) at 1000 g for 15 minutes at 4 ºC. The resulting supernatant or plasma was extracted and placed in 2 ml bijou tubes (Sarstedt Ltd, Leicester, UK) for storage at -20 ºC. Blood in silica gel vacutainers was allowed to clot in the fridge at 4 ºC for 24 hours. The resulting serum was then processed by the same method of centrifugation and storage as used for the plasma. Whole blood samples collected in the K_2EDTA vacutainers for subsequent haematological analysis, were transferred into 2 ml bijou tubes (Sarstedt Ltd, Leicester, UK), and stored at -20 ºC. Prior to any subsequent analysis plasma, serum and whole blood samples were removed from the freezer, allowed to defrost at room temperature, and vortexed mixed using an MT-20 vortex mixer (Philip Harris Ltd, Shenstone, UK) in order to obtain a uniform sample.

3.2.2 Haematological analysis

Whole blood samples collected in K_2EDTA vacutainers were analysed for haematological parameters including; white blood cells (WBC), monocyte numbers (Mon no), neutrophil numbers, (Neu No), lymphocyte number (Lym No), red blood cells (RBC), haematocrit percentage (HCT) and haemoglobin, (HGB) using a Vet animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK). Vacutainers were mixed using a Spiramix 5 (Demley Instruments Ltd, West Sussex, UK) in order to obtain a uniform sample before being analysed. The analysis of a control blood sample WD 1154 (ABX Diagnostics, Bedfordshire, UK) prior to experimental sample analysis was undertaken in order to ensure precision and accuracy.

3.2.3 Ceruloplasmin activity assay

Serum Cp activity was determined according to Henry *et al.* (1974) using a Cobas Miras plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). The 0.1 M pH 6 acetate buffer was prepared by dissolving 13.6 g of trihydrous sodium acetate (Fischer Scientific,

Loughborough, UK) in 900 ml of type-1 water. The resulting solution was then adjusted to pH 6 by adding 2.85 ml of glacial acetic acid diluted in 500 ml in type-1 water. The acetate buffer was also made up to 1 litre in type-1 water. The p-phenylenediamine (PPD) solution was prepared by dissolving 0.25 g of PPD (BDH Laboratories Supplies, Dorset, UK) in 100 ml of acetate buffer, and the pH adjusted to 6 using 0.1 M acetic acid (Sigma-Aldrich, Dorset, UK) and/or 0.1 M NaOH (Fischer Scientific, Loughborough, UK). Finally, the sodium azide solution was prepared by dissolving 1 g of sodium azide (Sigma-Aldrich, Dorset, UK) in 1 L of acetate buffer and subsequently adjusted to pH 6 using 0.1 M acetic acid (Sigma-Aldrich, Dorset, UK) and/or 0.1 M NaOH (Fischer Scientific, Loughborough, UK). Ceruloplasmin test (CPT) and ceruloplasmin blank (CPB) solutions were then prepared from these three solutions.

Each serum sample was pipetted into an individual Cobas Miras sample cup (ABX Diagnostics, Bedfordshire, UK) and placed on the reagent rack for subsequent analysis. This test utilises the oxidation of PPD by Cp to produce purple products with an absorption peak between 530 and 550 nm which can be measured spectrophotometrically (Henry *et al.*, 1974), as PPD can be oxidised by any Fe or Cu present in the serum, a blank (CPB) solution was also run in which sodium azide inhibits Cp activity. Ceruloplasmin activity was subsequently calculated as:

$$Ceruloplasmin\ activity\ (mg/dL) = CPT - CPB \qquad \text{Equation 3.6}$$

Where:

CPT = 20 ml of acetate buffer + 10 ml of PPD solution

CPB = 10 ml of acetate buffer + 10 ml of PPD solution + 10 ml of sodium azide solution

3.2.4 Superoxide dismutase assay

Whole blood SOD activity was determined according to Misra and Fridovich (1977) which had been adapted for use on the Cobas Miras Plus auto-analyser (Randox Laboratories, Antrim, UK, Kit catalogue no. SD125). This method operates on the principle that SOD inhibits free radicals generated when xanthine and xanthine oxidase react with 2- (4-iodophenyl)- 3-(4- nitrophenol)- 5- phenyl tetrazolium chloride to produce a red formazan dye. Sample superoxide dismutase activity determines the spectral absorption of this dye which can then be measured spectrophotometrically. Frozen whole blood samples were defrosted (to ensure cell lysis) and vortex mixed using an MT-20 vortex-mixer (Philip Harris

Ltd, Shenstone, UK). The samples were subsequently diluted 1:4 by pipetting 750 μL of type-1 water and 250 μL of defrosted blood sample into 2 ml bijou tubes (Sarstedt Ltd, Leicester, UK) and vortex mixed. These samples were then further diluted 1:50 by adding 490 μL of 0.01 mol/L phosphate buffer (pH 7.0; Randox Laboratories, Antrim, UK, kit catalogue number SD124) and 10 μL of previously diluted sample into Cobas Miras sample cups (ABX, Diagnostics, Bedfordshire, UK). These cups were then vortexed mixed and placed on the reagent rack for analysis by the Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). The analysis of a control blood sample (Randox Laboratories, Antrim, UK, kit catalogue no. SD126) prior to experimental sample analysis was undertaken in order to ensure precision and accuracy.

3.2.5 Blood metabolites and acute phase proteins

Plasma samples were analysed for glucose, urea, and β-hydroxybutyrate (BHB) in experiment 1 (Kit catalogue no. GL 1611, UR221, RB1008; Randox Laboratories, Antrim, UK), AST and GGT in experiment 2 (Kit catalogue no. AS1202, GT553; Randox Laboratories, Antrim, UK), and glucose, BHB, and non-esterified fatty acids (NEFA) in experiment 3 (Kit catalogue no. GL1611, RB1008, FA115; Randox Laboratories, Antrim, UK). Serum samples were analysed for haptoglobin (Hp) in experiment 1 (Kit catalogue no. TP801; Tridelta Development Ltd, Kildare, Republic of Ireland), and GLDH (Kit catalogue no. GL441; Randox Laboratories, Antrim, UK) in all three experiments. The Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK) was used for all blood metabolite and acute phase protein analyses.

3.2.6 Plasma trace element analysis

Plasma samples were diluted 1:50 in an acidic diluent which contained 0.50 % concentrated HNO_3 (Fischer Scientific, Loughborough, UK), 2.00 % HPLC grade methanol (Sigma-Aldrich, Dorset, UK), and 0.05 % Triton X-100 (Fischer Scientific, Loughborough, UK) in type-1 water in procedure adapted from Cope *et al.* (2009), with modifications from Pruszkowski and Neubauer (2017). These included an increase in the dilution factor from 1:20 to 1:50 (Pruszkowski and Neubauer, 2017), and a change in acidic diluent constituents from the original composition (2.00 % HNO_3, 0.5% HPLC methanol, and 0.1% Triton X-100) quoted by Cope *et al.* (2009). This was to ensure maximum element recovery in the Nexion 2000 ICP-MS (Perkin Elmer, Beaconsfield, UK; Pruszkowski and Neubauer, 2017). Blank and calibration standards were prepared containing 0, 10, 50, 100, 200, and 400 μg/kg of

the minor elements Cu, Mo, Fe and Zn (TraceCert, Sigma-Aldrich, Dorset, UK) in a solution of 0.50 % concentrated HNO_3, 2.00 % HPLC grade methanol, and 0.05 % Triton-X 100. Dilute plasma samples were subsequently analysed for Cu, Mo, Fe and Zn by ICP-MS (Perkin Elmer, Beaconsfield, UK). The ClinCheck certified lyophilised plasma control sample 2 (Product no. 8885 RECIPE; Chemicals and Instruments GmbH, Munich, Germany) was analysed in order to ensure precision and accuracy of trace element determination (Table 3.2).

3.3 Biopsy sample collection and hepatic trace element analysis

Liver biopsy samples were collected via the 11th intercostal space (about 20 cm below the tip of the transverse process) using the procedure described by Davis and Jebbet (1981). Local anaesthetic (Adrenacaine; Norbrook Laboratories, Newry, UK) was used to numb the infiltration site, and a 10 mm incision was made through the thickness of the intercostal wall. The cannula (containing the cannula) was inserted through the incision and directed towards the liver, and the trocar was then withdrawn. The cannula was then advanced through the liver three to four times to collect sufficient sample. After removing the cannula from the animal, the trocar was re-inserted into the cannula to expel the liver biopsy sample. Additionally, after withdrawal of the cannula, terramycin aerosol spray (Zoetis UK, Surrey, UK) was applied to the wound and it was closed through the use of surgical staples, and a broad-spectrum antibiotic was administered to prevent infection (Excenel; Zoetis UK, Surrey, UK). Biopsy samples were then immediately snap frozen in liquid nitrogen and subsequently stored at -80 ºC. Liver samples were defrosted at room temperature and approximately 0.50 g (0.25 to 0.75 g; exact weight recorded to 4 decimal places) of fresh liver sample was accurately weighed into a 50 ml DigiPREP tube (QMX Laboratories, Essex, UK). The samples were then oven (Binder, Cole-Palmers, UK) dried at 60 ºC to a constant weight, re-weighed, 6 ml of concentrated HNO_3 (Fischer Scientific, Loughborough, UK) added, and then left overnight in an oven (Binder, Cole-Palmers, UK) at 60 ºC to digest as described by Sinclair *et al.* (2013). Samples were then allowed to cool to room temperature and made up to 50 ml with type-1 water.

Digested liver samples were then diluted 1:50 in an acidic diluent which contained 0.50 % concentrated HNO_3 (Fischer Scientific, Loughborough, UK), 2.00 % HPLC grade methanol (Sigma-Aldrich, Dorset, UK), and 0.05 % Triton X-100 (Fischer Scientific, Loughborough, UK) in type-1 water in a procedure adapted from Cope *et al.* (2009) according to Pruszkowski and Neubauer (2017) as described in Section 3.2.6. Blank and calibration

standards were prepared containing 0, 10, 50, 100, 200, and 400 μg/kg of the minor elements Cu, Mo, Fe and Zn (TraceCert, Sigma-Aldrich, Dorset, UK), in a solution of 0.50 % concentrated HNO_3, 2.00 % HPLC grade methanol, and 0.05 % Triton-X 100. Diluted liver samples were subsequently analysed for Cu, Mo, Fe and Zn by inductively coupled plasma mass spectrometry (Nexion 2000; Perkin Elmer, Beaconsfield UK). The certified EU reference liver sample BCR-185 (Sigma-Aldrich, Dorset, UK) was routinely extracted and analysed in order to ensure precision and accuracy (Table 3.2).

Table 3.2. Certified plasma and liver reference material results of ICP-MS analyses.

	Certified reference material[1]					
	Lyophilised plasma control 2			EU BCR-185, liver		
Element	Certified concentration	Analysed concentration	%[2]	Certified concentration	Analysed concentration	%[2]
	μmol/L			mg/kg DM		
Cu	19.9 ± 4.2	18.0 ± 0.5	90	277 ± 5	278 ± 2	100
Mo	0.06 ± 0.02	0.06 ± 0.01	110			
Fe	20.7 ± 3.1	19.1 ± 0.5	108			
Zn	29.9 ± 4.5	28.9 ± 0.9	97	138.6 ± 2.1	137.0 ± 3.5	99

[1] Uncertainties expressed as the half-width of the 95% confidence interval of the mean.
[2] Percentage recovered = %.

CHAPTER 4: Dietary starch concentration alters rumen pH, hepatic copper retention, and performance in lactating Holstein-Friesian dairy cows receiving added dietary sulfur and molybdenum

4.1 Introduction

Copper is an essential trace element, which primarily functions as an enzyme co-factor within the animal (Linder and Hazegh-Azam, 1996; Davis and Metz, 1987). Clinical Cu deficiency has consequences for dairy cow health, performance and fertility, and is characterised by symptoms including impaired growth, reduced fertility and anaemia (McDowel, 1985; Suttle et al., 1987). A deficiency of Cu is rarely a result of a lack of supply within the diet, but is more commonly related to interactions with dietary antagonists such as S, Mo, Fe, and Zn which inhibit Cu absorption (Suttle, 2010). It has been reported that dietary sulfates are reduced to sulfides within the rumen (Dick et al., 1975), and subsequently react with molybdate to form thiomolybdates which form an insoluble complex with Cu preventing its absorption (Suttle, 1991). It is recognised that thiomolybdates may be present in the rumen as mono-, di-, tri-, or tetrathiomolybdates (Gould and Kendall, 2011), whereby molybdate sequentially reacts with sulfide to form tetra-thiomolybdate as the end product (Suttle, 1991). Clarke and Laurie (1980) identified thiomolybdate speciation to be highly pH-dependent with greater proportion of tetrathiomolybdate formation at lower pH values. When tri- and tetrathiomolybdates are absorbed into the bloodstream they have the ability to bind and impair the function of Cu containing enzymes such as Cp which may subsequently impact upon animal health and performance (Gould and Kendall, 2011). Thiomolybdate formation in the rumen can also be affected by basal forage source, although the understanding of the mechanisms surrounding this remain poor (Suttle, 1983; Sinclair et al, 2017). Sinclair *et al.* (2017) noted a decrease in DMI, milk yield, and hepatic Cu concentration in response to S and Mo supplementation when cows were fed a grass silage- compared to a maize silage-based diet, and it was suggested that this may have been due to the effect of rumen pH on S metabolism and/or thiomolybdate formation.

It is well established that the fermentable carbohydrate content of the forage can influence rumen pH (Taayab *et al.*, 2018; Hassanat *et al.*, 2013). Starch is a rapidly fermentable carbohydrate which can decrease rumen pH via the production of organic acids (Firkins, 1997), particularly when the buffering capacity of the diet is low (Maekawa *et al.*, 2002). A large body of evidence exists regarding the effect of dietary starch on milk yield, fat and protein content (Gómez *et al.*, 2016). However little evidence is available regarding the

effect of dietary starch content on Cu metabolism in lactating Holstein-Friesian dairy cows, despite potential links between forage starch levels, rumen pH, and thiomolybdate formation (Gould and Kendall, 2011; Taayab *et al.*, 2018). This lack of understanding regarding the effect of dietary factors on Cu metabolism may be contributing to unnecessary over-supplementation of the element within dairy rations. There have been a number of surveys in the UK and USA (Sinclair and Atkins, 2015; Castillo *et al.*, 2013), that have reported on farm Cu supplementation to be well in excess of that recommended by feed standards such as NRC (2001) or ARC (1980). A consequence of this over-supplementation is that 38% of dairy cull cows within the UK are reported to have hepatic Cu concentrations above the 508 mg Cu/kg DM limit thought to pose a risk of clinical Cu toxicity (Kendall *et al.*, 2015). Feeding Cu above the animal's requirements can also result in clinical Cu poisoning and death, whereby high hepatic Cu concentrations lead to lysosome rupture, hepatic necrosis, and death (Bidewell *et al.*, 2000). The objectives of this study were to determine the effect of dietary starch level when fed with or without added S and Mo on indicators of Cu status and performance in lactating Holstein-Friesian dairy cows.

4.2 Materials and Methods

4.2.1 Animals, Treatments, housing, and Management

All procedures involving animals undertaken in this study were conducted in accordance with the United Kingdom (UK) Animals (Scientific Procedures) Act 1986 (UK Parliament, amended 2012), and received local ethical approval. Sixty Holstein-Friesian dairy cows (48 multiparous and 12 primiparous) that were 33 (SE ± 2.5) days post calving, with a live weight of 659 (SE ± 17.7) kg, and yielding 41 (SE ± 0.9) kg were used. Based upon recordings taken in the week prior to allocation, cows were blocked and allocated to 1 of 4 dietary treatments according to calving date, parity (multiparous or primiparous), milk yield, and BCS (5-point scale with 0.25 increments; Ferguson *et al.*, 1994). Cows remained on study for a total of 98 days. From calving until week 5 of lactation all cows were group housed and fed a TMR that contained (g/kg DM); 374 maize silage; 118 lucerne silage; 86 Sweet Starch[1]; 79 soy hulls: 73 rapeseed meal; 73 wheat distiller's dark grains; 60 grass silage; 42 Proflo Syrup[2]; 31 Soy Pass[3], 20 palm kernel meal; 13 chopped wheat straw; 9 protected fat; 7 minerals and vitamins; 6 molasses; 4 salt; 4 Acid Buf[4]; and 1 of SC digestaid[5]. Animals

[1] Baking industry co-product blend, KW Alternative Feeds, Staffordshire, UK.
[2] Wheat Vinasses, KW Alternative Feeds, Staffordshire, UK.
[3] Rumen protected soya bean meal, KW Alternative Feeds, Staffordshire, UK.
[4] Ground calcareous marine algae, KW Alternative Feeds, Staffordshire, UK.
[5] Live yeast, Lallemand Inc, Malvern, UK.

were blocked and randomly allocated to 1 of 4 dietary treatments based on parity (multiparous or primiparous) and the milk yield recorded in the week prior to allocation. Cows remained on study for a total of 98 days (14 weeks).

Four diets were formulated to contain a maize: grass silage ratio of 1:1 (DM basis), 15 mg Cu/kg DM, and a dietary starch concentration of 220 (HS) or 130 g/kg DM (LS; Table 4.1). In order to examine the effects of Cu antagonists, the diets were either un-supplemented (-) or supplemented (+) with added S and Mo. Additional Cu was supplied as copper sulfate pentahydrate ($CuSO_4.5H_2O$), sulfur as flowers of sulfur, and molybdenum as sodium molybdate (Rumenco, Staffordshire, UK). The supplemented diets were formulated to contain a total dietary concentration of approximately 3.0 g S/kg DM and 5.5 mg Mo/kg DM, whereas un-supplemented diets were formulated to contain approximately 2.5 g S/kg DM and 1.5 mg Mo/kg DM. The 4 dietary treatments were therefore: LS- [130 g/kg DM starch, no additional antagonists]; LS+ [130 starch, with additional S and Mo]; HS- [220 g/kg DM dietary starch, no additional antagonists]; HS+ [220 g/kg DM dietary starch, with additional S and Mo]. All 4 diets were formulated to support a milk yield of approximately 38 kg/d according to Thomas (2004). Dietary ingredients were mixed using a Hi-spec forage mixer calibrated to ± 0.1 kg and fed as a TMR through roughage intake feeders (Hokofarm, Marknesse, Netherlands), fitted with an automatic weighing and animal identification system calibrated to ± 0.1 kg (Sinclair et al., 2007). Fresh feed was offered on a daily basis at 105% of ad-libitum intake, and refusals collected three times per week on a Monday, Wednesday and Friday. Cows were housed in free stalls fitted with foam mattresses in the same area of an open span building. Stalls were bedded three times per week with a sawdust-lime mix, and passageways were scraped by automatic scrapers. All cows had continual access fresh water containing 13.4 mg/L, 0.8 µg/L, 4.6 µg/L, and 0.2 µg/L of S, Cu, Fe, and Mo respectively.

Table 4.1. Diet composition and chemical analysis of low (LS) and high starch (HS) rations fed either without (-) or with (+) added S and Mo.

Item	LS-	LS+	HS-	HS+
Ingredient, g/kg DM				
Grass silage	264	264	265	265
Maize silage	264	264	265	265
Rolled wheat	13	13	212	212
Soy pass[1]	42	42	--	--
Soy hulls	157	157	59	59
Molassed sugar beet feed	114	114	--	--
Soy bean meal	59	59	86	86
Rapeseed meal	29	28	43	43
Wheat distiller's dark grains	29	29	43	43
Palm kernel meal	8	8	12	12
Molasses	3	3	3	3
Limestone	--	1	--	1
Salt	1	1	1	1
Protected fat	8	8	2	2
Mins/vits[2/3]	9[2]	9[3]	9[2]	9[3]
Total	1000	1000	1000	1000
Chemical analysis				
DM, g/kg	430	426	429	423
Crude protein, g/kg DM	166	164	167	166
Ash, g/kg DM	78	79	68	70
Organic matter, g/kg DM	922	921	932	930
NDF, g/kg DM	435	441	376	374
ADF, g/kg DM	273	267	229	223
Ether extract, g/kg DM	22	23	24	22
Starch, g/kg DM	149	148	224	225
WSC, g/kg DM	22	21	17	17
Gross energy[4], MJ/kg DM	18.7	18.7	19.0	19.0
Metabolisable energy[4], MJ/kg DM	12.0	12.0	12.1	12.1
Ca, g/kg DM	6.77	5.26	6.79	5.31
P, g/kg DM	4.03	4.04	4.14	4.20
Mg, g/kg DM	2.28	2.20	2.25	2.17
S, g/kg DM	2.25	3.04	2.20	2.96
Cu, mg/kg DM	15.1	14.9	14.8	14.6
Mo, mg/kg DM	1.1	5.7	1.1	5.5
Zn, mg/kg DM	70.6	71.4	73.0	68.1
Fe, mg/kg DM	248	242	252	234
Mn, mg/kg DM	62.4	65.9	66.4	59.0

[1]Rumen protected soya bean meal, KW Alternative Feeds, Staffordshire, United Kingdom).
[2]LS- and HS- mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 180, P 53, Mg 75, Na 75, S 0; Trace minerals (mg/kg): Cu 825, Zn 4,500, Mn 1,500, I 300, Co 30, Se 26 and Mo 0; vitamins (iu/kg) were: retinol 225, 000, cholecalciferol 75,000, and all *rac* α-tocopherol acetate 3,000.
[3]LS+ and HS+ mineral/vitamin premix (Rumenco, Staffordshire, UK). Major minerals (g/kg): Ca 148, P 53, Na 75, Mg 75, S 140; Trace minerals (mg/kg): Cu 825, Zn 4,500, Mn 1,500, I 300, Co 30, Se 26, and Mo 488; vitamins (iu/kg) were: retinol 225, 000, cholecalciferol 75,000, and all *rac* α-tocopherol acetate 3,000.
[4] Calculated using standard and declared values (Andrew, 1991; MAFF, 1982; Sauvant *et al.*, 2002).

4.2.2 Experimental Routine

Cows were milked twice daily through a Westaflia 40-point internal rotary parlour at approximately 0600 and 1600 h. The milk yield of each cow was recorded at each milking, with samples taken on a fortnightly basis at consecutive morning and afternoon milkings, for subsequent analyses of milk composition and somatic cell count (SCC). Cows were weighed and body condition scored (Ferguson *et al.*, 1994) before afternoon milking in the week prior to allocation, and on a fortnightly basis afterward. Reticular pH boluses (eCow Ltd, Devon, UK) were administered to 24 cows (six per treatment) in the week prior to commencing the study, with reticular pH recorded at 15-minute intervals. Forage samples were collected on a weekly basis, dried to constant weight, and the maize to grass silage adjusted to achieve the desired ratio. Fresh samples of the 4 diets were collected immediately post-feeding on a weekly basis and stored at -20^{o}C prior to subsequent analysis. Blood samples were collected via the jugular vein at 1100 h during weeks 0, 1, 2, 4, 6, 10, and 14 of the study into vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) containing lithium heparin (to determine urea and β-hydroxybutyrate; BHB), silica gel (to determine haptoglobin and ceruloplasmin; Hp and Cp), fluoride/oxalate (to determine glucose), dipotassium ethylenediaminetetraacetic acid (K_2EDTA; to determine haematological profile), and potassium ethylenediaminetetraacetic acid (K_3EDTA; to determine mineral concentrations). Liver biopsy samples were collected during weeks 0 and 14 of the study via the 11th intercostal space as described by Davies and Jebbett (1981), immediately snap frozen in liquid nitrogen and stored at -80^{o}C prior to subsequent analysis.

4.2.3 Chemical analysis

Weekly diet samples were bulked on a monthly basis and analysed according to AOAC (2012) for DM (934.01; intra-assay CV of 1.5%), crude protein (CP; 990.03; Section 3.1.1; intra-assay CV of 1.6%), ether extract (EE; 2003.05; Section 3.1.5; intra-assay CV of 5.3%), and ash (942.05; Section 3.1.2; intra-assay CV of 0.8%). Samples were also analysed for starch (Section 3.1.6; intra-assay CV of 3.3%) according to ISO 6493 (2000) at Sciantic Analytical (Stockbridge Technology Centre, North Yorkshire, UK). The neutral detergent fibre (NDF; Section 3.1.3) and acid detergent fibre (ADF; Section 3.1.4) content of the diets were determined according to Van Soest *et al.* (1991), heat-stable was used α-amylase for NDF determination (Sigma Aldrich, Dorset, UK; intra-assay CV of 1.2 % for NDF and 2.7% for ADF respectively). The water-soluble carbohydrate (WSC; Section 3.1.7) of the diets was determined according to Thomas (1977; intra-assay CV of 3.0%). Dietary minerals were extracted using a DigiPREP digestion system (QMX Laboratories, Essex, UK), and

analysed by inductively coupled plasma-mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield, UK; Section 3.1.8) as described by Sinclair and Atkins (2015). Plasma samples were analysed for urea, BHB, and glucose (Randox Laboratories, Antrim, UK; kit catalogue no. UR221, RB1007, GL1611; Section 3.2.5; intra-assay CV of 2.6%, 4.0%, and 0.9% respectively). Serum samples were analysed for Cp according to Henry *et al.* (1974; Section 3.2.3; intra-assay CV of 0.9%), and Hp (Tridelta Development Ltd, Kildare, Republic of Ireland; kit catalogue no. HP801; Section 3.2.5; intra-assay CV of 0.8%). Whole blood samples were analysed for SOD activity (Randox Laboratories, Antrim, UK; kit catalogue no. SD 125; intra-assay CV of 3.3%). The analysis of all plasma, serum, and whole blood samples was conducted using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Haematological parameters were analysed using a Vet Animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK; Section 3.2.2). Liver samples were digested overnight at 60^{o}C in concentrated nitric acid. Plasma and liver samples were analysed for Cu, Fe, Zn and Mo by ICP-MS (Nexion 2000; Perkin Elmer, Beaconsfield, UK; Sections 3.2.6 and 3.3 intra-assay CV of 0.5%, 1.4%, 2.2%, and 1.2% for plasma; intra-assay CV of 4.1%, 3.0%, 2.6% and 2.3% for liver respectively). Milk samples were analysed for fat, protein, lactose, urea and SCC by Eurofins Laboratories (Wolverhampton, UK).

4.2.4 Calculations and statistical analysis

Milk (MJ/kg) energy content was calculated according to Tyrrel and Reid (1965) using the equation:

$$\text{Milk energy (MJ/kg)} = (\text{F} \times 0.0384) + (\text{P} \times 0.0223) + (\text{L} \times 0.0199) - 0.108$$

Equation 4.1

Where, F = milk fat content (g/kg), P = milk protein content (g/kg), and L = milk lactose content (g/kg). Mean daily ME balance (MEb; MJ/cow/d) for each animal was calculated according to Thomas (2004) using the equation:

$$\text{MEb (MJ/cow/d)} = \left[(\text{M}_{\text{Ml}} \times \text{LW}^{0.75}) + \left(\frac{0.0013 \times \text{LW}}{\text{K}_{\text{m}}}\right) - 10\right] - \text{Mei}$$

Equation 4.2

Where, M_{ml} = ME required for maintenance and milk production (MJ/kg of metabolic live weight), $LW^{0.75}$ = metabolic live weight, K_m = ME utilisation efficiency (calculated as 0.35 x ME/gross energy + 0.503), and MEi = ME intake (MJ/cow/d).

Continuous performance and blood parameters were analysed as a 2 x 2 factorial design using a repeated measures ANOVA. The treatment degrees of freedom were split into main effects of dietary starch level (S; low (LS) versus high (HS)), antagonist (A; with (+) versus without (-)), and their interaction (Int), and analysed as:

$$Y_{ijkl} = \mu + B_i + S_j + A_k + T_l + S.A_{jk} + S.T_{jl} + A.T_{Kl} + S.A.T_{jkl} + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; S_j = effect of starch level (j = low or high); A_k = effect of S and Mo (k= with or without); T_l= effect of time $S.A_{jk}$ = interaction between dietary starch levels and antagonists; $A.T_{kl}$ = interactions between dietary antagonists and time; $S.A.T_{jkl}$ = interaction between starch level, antagonists, and time, and ε_{ijkl} = residual error.

Non-continuous performance parameters including liver mineral concentrations were analysed as a 2 x 2 factorial design ANOVA as:

$$Y_{ijkl} = \mu + B_i + S_j + A_k + S.A_{jk} + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of starch level (j = low or high); A_k = effect of antagonist; $S.A_{jk}$ = interaction between starch level and antagonist, and ε_{ijkl} = residual error. Milk SCC was transformed to $\log_{10}$ prior to analysis, and live weight change was determined by linear regression of fortnightly live weight measurements. All statistical analyses were conducted using Genstat version 18 (VSN International, Ltd, Oxford, UK), and means are presented with their associated standard error of the difference of the mean. $P < 0.05$ was considered the threshold for significance, with $P < 0.1$ denoting a trend.

4.3 Results

4.3.1 Dietary analysis, reticular pH, and animal performance

The four treatment diets had similar DM, CP and EE contents, with mean values of 427 g/kg, 166, and 23 g/kg DM respectively (Table 4.1). Mean dietary NDF and ADF values were 63, and 49 g/kg DM lower in low compared to the high starch diets respectively (LS- and LS+). The mean dietary starch contents of the high starch diets (HS- and HS+) was 76 g/kg DM higher than the low starch diets (LS- and LS+), with mean dietary concentrations of 149 g/kg DM for the low starch diets (LS- and LS+), and 225 g/kg DM for the high starch diets (HS- and HS+). Mean WSC concentrations were also 5 g/kg DM higher in the high (22 g/kg DM; HS- and HS+) compared to the low starch diets (17 g/kg DM; LS- and LS+). All

four diets had similar concentrations of Mg and P with mean values of 2.23 and 4.10 g/kg DM respectively. In contrast, the mean dietary Ca concentration of the control diets (LS- and HS-) was 1.50 g/kg DM higher than the diets containing additional antagonists (LS+ and HS+). There were similar dietary Cu concentrations across all four diets with a mean value of 14.9 mg/kg DM. Both diets with added antagonists (LS+ and HS+) had S and Mo concentrations (mean values of 3.00 g/kg DM and 5.6 mg/kg DM) that were 0.78 g/kg DM and 4.5 mg/kg DM higher than the unsupplemented diets respectively (LS- and HS-; mean values of 2.23 g/kg DM and 1.1 mg/kg DM).

Reticular pH was at its highest immediately prior to feeding with a subsequent decline across all treatments thereafter reaching a nadir at approximately 18:00 h ($P < 0.001$; Figure 4.1). An effect of dietary starch level on reticular pH was also noted, with cows receiving the high starch diets having a mean reticular pH that was 0.2 pH units lower ($P < 0.05$) than those fed low starch diets (6.23 vs. 6.38 for HS and LS respectively; Table 4.2). Cows fed the high starch diets had a daily minimum reticular pH that was 0.2 pH units lower ($P < 0.01$), and spent a greater ($P < 0.05$) percentage of time below pH 5.8 (90 vs. 1 min/d) compared to cows fed low starch diets. There was no effect ($P > 0.05$) of additional S and Mo on any reticular pH parameter.

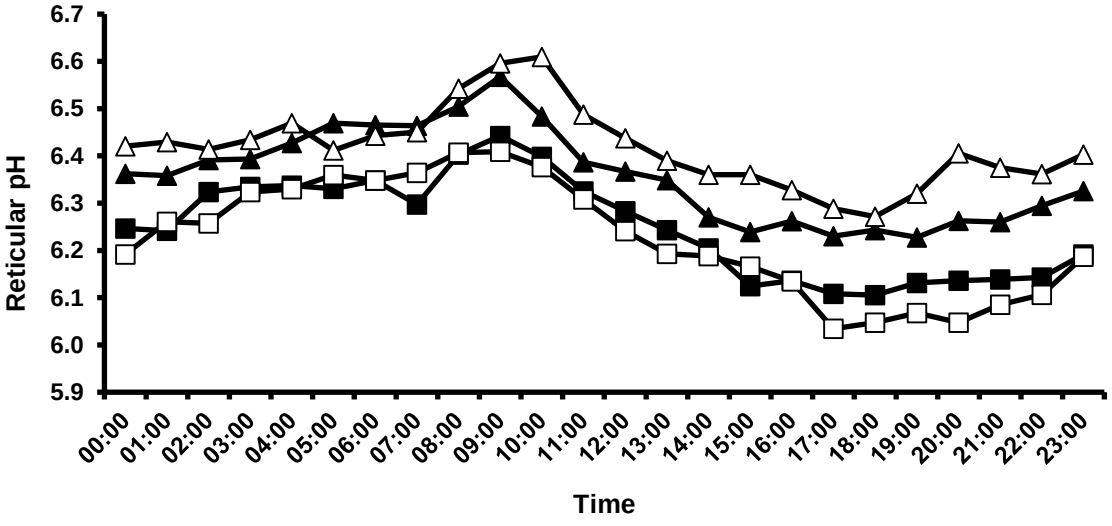

Figure 4.1. Reticular pH of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. Pooled SED = 0.094. Starch, P = 0.022; antagonist, P = 0.755; starch x antagonist, P = 0.487; time, P < 0.001, time x starch, P = 0.256; time x antagonist, P = 0.601, time x starch x antagonist, P = 0.305. Cows were fed at 08:00.

Table 4.2. Reticular pH of dairy cows fed low (LS) or high starch (HS) diets without (-) or with (+) added S and Mo.

	Diet					P-value[1]		
	LS-	LS+	HS-	HS+	SED	S	A	Int
Daily minimum pH	5.97	6.05	5.86	5.76	0.088	0.008	0.856	0.168
Daily maximum pH	6.72	6.77	6.63	6.67	0.096	0.199	0.513	0.914
Mean pH	6.35	6.41	6.24	6.22	0.079	0.022	0.755	0.487
% time < 5.8 pH[2]	0.09	0.00	3.50	9.04	4.086	0.048	0.404	0.316
% time < 6.0 pH	4.96	4.01	19.00	23.6	8.504	0.017	0.769	0.655
% time < 6.2 pH	24.0	22.9	43.5	40.9	12.01	0.050	0.828	0.932
% time < 6.5 pH	73.9	60.7	78.5	78.6	9.90	0.137	0.370	0.365

[1]S = main effect of dietary starch level, A = main effect of dietary antagonists. Int = interaction between dietary starch level and antagonists.
[2]percentage of each day spent below pH level, data not normally distributed.

The addition of S and Mo reduced (P < 0.001) mean daily DMI by 1.8 kg DM/d (Table 4.3), an effect that was evident from week 2 of the study (Figure 4.2). There was also a trend (P < 0.1) for an interaction between dietary starch concentration and Cu antagonists, where the addition of S and Mo decreased DMI to a greater extent when cows were fed high compared to low starch diets. Mean milk yield decreased (P < 0.001) across all dietary treatments throughout the duration of the study but there was no effect (P > 0.05; Figure 4.3) of dietary starch concentration, Cu antagonists or interaction between starch and antagonists (Table 4.3). There was no effect (P > 0.05) of dietary treatment on milk fat, lactose, or SCC content, although there was an effect (P < 0.05) of dietary starch concentration on milk protein content, protein yield, and milk urea content which were 2.8 g/kg, 0.09 kg/d and 2.1 mg/kg higher in cows fed the high starch diets respectively. In contrast, there was no effect (P > 0.05) of dietary treatment on live weight, daily live weight change, BCS, or BCS change. Despite cows fed the antagonist supplemented diets having a lower (P < 0.001) ME intake of 268 MJ/cow/d compared to 246 MJ/cow/d for cows fed the supplemented diets, there was no difference (P > 0.05) in ME output between the dietary treatments. The addition of S and Mo also increased (P < 0.001) the positive energy balanced experienced by cows from 1 to 20 MJ/cow/d.

Table 4.3. Intake and performance in dairy cows fed low (LS) or high starch (HS) diets without (-) or with (+) added S and Mo.

	Diet					P-value[1]		
	LS-	LS+	HS-	HS+	SED	S	A	Int
Intake								
DM, kg/d	21.8	20.9	22.5	19.9	0.70	0.698	0.001	0.072
ME, MJ/cow/d	262	252	273	240	8.5	0.971	0.001	0.069
Milk yield, kg/d	38.2	36.7	37.1	35.8	1.39	0.330	0.159	0.926
Fat, g/kg	39.5	42.0	40.8	41.2	1.22	0.795	0.241	0.400
Protein, g/kg	29.9	31.4	33.8	33.0	0.78	<0.001	0.549	0.048
Lactose, g/kg	46.7	46.3	46.2	45.9	0.42	0.150	0.276	0.950
Milk energy, MJ/kg	3.00	3.13	3.13	3.12	0.078	0.243	0.306	0.230
Milk energy output, MJ/cow/d	115	113	117	111	3.9	0.889	0.222	0.398
Urea, mg/dL	22.2	23.6	24.5	25.4	1.35	0.034	0.237	0.780
Fat yield, kg/d	1.52	1.51	1.51	1.49	0.037	0.756	0.774	0.799
Protein yield, kg/d	1.14	1.14	1.27	1.18	0.033	0.004	0.077	0.100
Lactose yield, kg/d	1.78	1.70	1.75	1.64	0.066	0.324	0.060	0.823
Milk SCC, log_{10}/mL	1.66	1.39	1.43	1.58	0.108	0.799	0.421	0.010
Live weight, kg	654	654	654	649	24.7	0.877	0.881	0.905
Δ live weight[2], kg/d	0.10	0.23	0.24	0.36	0.117	0.130	0.149	0.987
Condition score	2.57	2.65	2.67	2.65	0.099	0.087	0.619	0.794
Δ condition score[2]	-0.15	-0.08	-0.05	-0.01	0.082	0.130	0.347	0.859
ME balance, MJ/cow/d	+1	+12	+1	+28	6.6	0.103	<0.001	0.074

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists; [2] Wks 0-14.

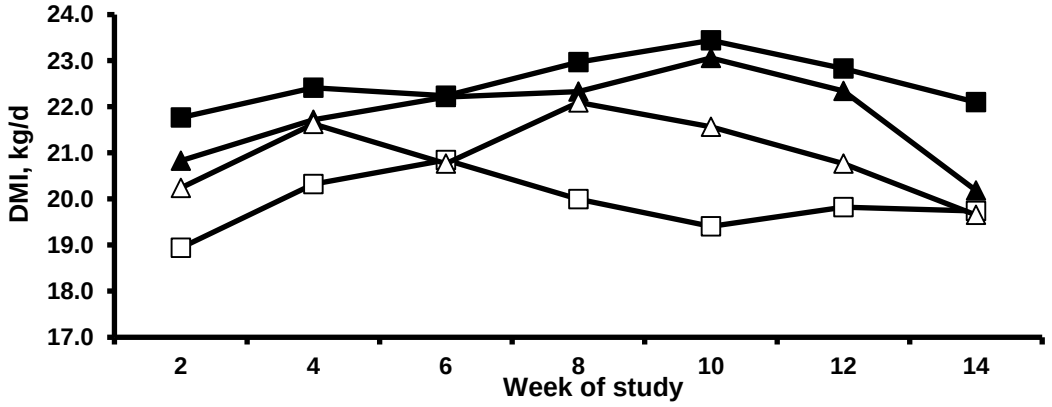

Figure 4.2. Fortnightly dry matter intake (DMI) in early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. Pooled SED = 0.96. Starch, P = 0.698; antagonist, P = 0.001; starch x antagonist, P = 0.072; time, P < 0.001; time x starch, P = 0.136; time x antagonist, P = 0.228; time x starch x antagonist, P = 0.296.

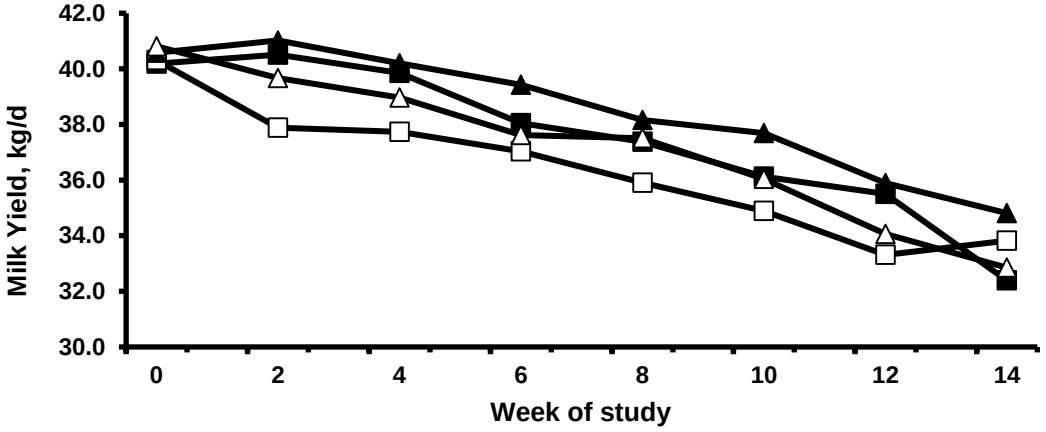

Figure 4.3. Fortnightly milk yield of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. Pooled SED = 1.59. Starch, P = 0.330; antagonist, P = 0.159; starch x antagonist, P = 0.926; time, P < 0.001; time x starch, P = 0.887; time x antagonist, P = 0.372; time x starch x antagonist, P = 0.136.

4.3.2 Plasma mineral profile, blood metabolites, cu-mediated enzymes, and acute phase proteins

There was no effect (P > 0.05) of dietary treatment on plasma Cu concentration, with a mean value of 15.3 µmol/L (Table 4.5), but there was an effect of time (P < 0.001) with concentrations increasing until week 2 across all treatments before decreasing at week 4 and fluctuating thereafter (Figure 4.4a). Cows fed the antagonist supplemented diets (LS+ and HS+) had higher (P < 0.01) plasma Mo concentrations than those fed the unsupplemented diets. There was also an interaction (P < 0.05) between dietary starch concentrations and Cu antagonist supplementation, where plasma Mo concentrations increased to a greater extent when cows were fed high starch (0.6 µmol/ L) compared to low starch diets (0.4 µmol/ L; Figure 4.4b). Neither plasma Zn or Fe concentrations were affected (P > 0.05) by dietary treatment, with mean values of 14.4 and 42.1 µmol/L respectively. Serum Cp activity was higher (P < 0.01) in cows fed the high starch diets, but there was no effect (P > 0.05) of dietary treatment on whole blood SOD. Cows fed the high starch diets also had higher (P < 0.001) Cp: plasma Cu ratios than those fed the low starch diets. There was also an effect (P < 0.01) of dietary starch concentration on plasma urea which was 0.65 mmol/L higher in cows fed the high starch diets. In contrast there was no effect of dietary treatment (P > 0.05) on plasma glucose or BHB, with mean values of 3.47 and 0.55 mmol/L respectively. There was also no effect (P > 0.05) of dietary starch concentration or antagonist supplementation on serum Hp with a mean of 0.31 mg/mL across all four dietary treatments.

Table 4.4. Plasma mineral concentrations, blood metabolites and ceruloplasmin in dairy cows fed low (LS) or high starch (HS) diets without (-) or with (+) added S and Mo.

	Diet					P-value[1]		
Item[2]	LS-	LS+	HS-	HS+	SED	S	A	Int
Plasma Cu, µmol/L	15.5	15.6	14.6	15.6	0.80	0.387	0.324	0.454
Plasma Mo, µmol/L	0.22	0.64	0.32	0.89	0.049	0.004	<0.001	0.037
Plasma Fe, µmol/L	40.8	42.0	42.0	43.5	1.94	0.341	0.323	0.895
Plasma Zn, µmol/L	14.6	14.2	14.1	14.7	0.56	0.959	0.708	0.229
SOD U/ g of Hb[2]	2377	2444	2519	2347	135.3	0.823	0.502	0.214
Cp, mg/ dL	18.5	17.5	21.2	22.6	1.57	0.001	0.872	0.288
Cp; Cu	1.16	1.14	1.34	1.42	0.076	<0.001	0.601	0.362
BHB, mmol/L	0.60	0.53	0.49	0.58	0.068	0.511	0.796	0.122
Glucose, mmol/L	3.41	3.43	3.62	3.43	0.095	0.133	0.234	0.113
Urea, mmol/L	3.70	3.66	4.33	4.32	0.285	0.003	0.914	0.924
Hp, mg/mL	0.37	0.25	0.24	0.38	0.116	0.951	0.888	0.118

[1]S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.
[2]Blood samples were collected during weeks 0, 1, 2, 4, 6, 10 and 14 of the study.

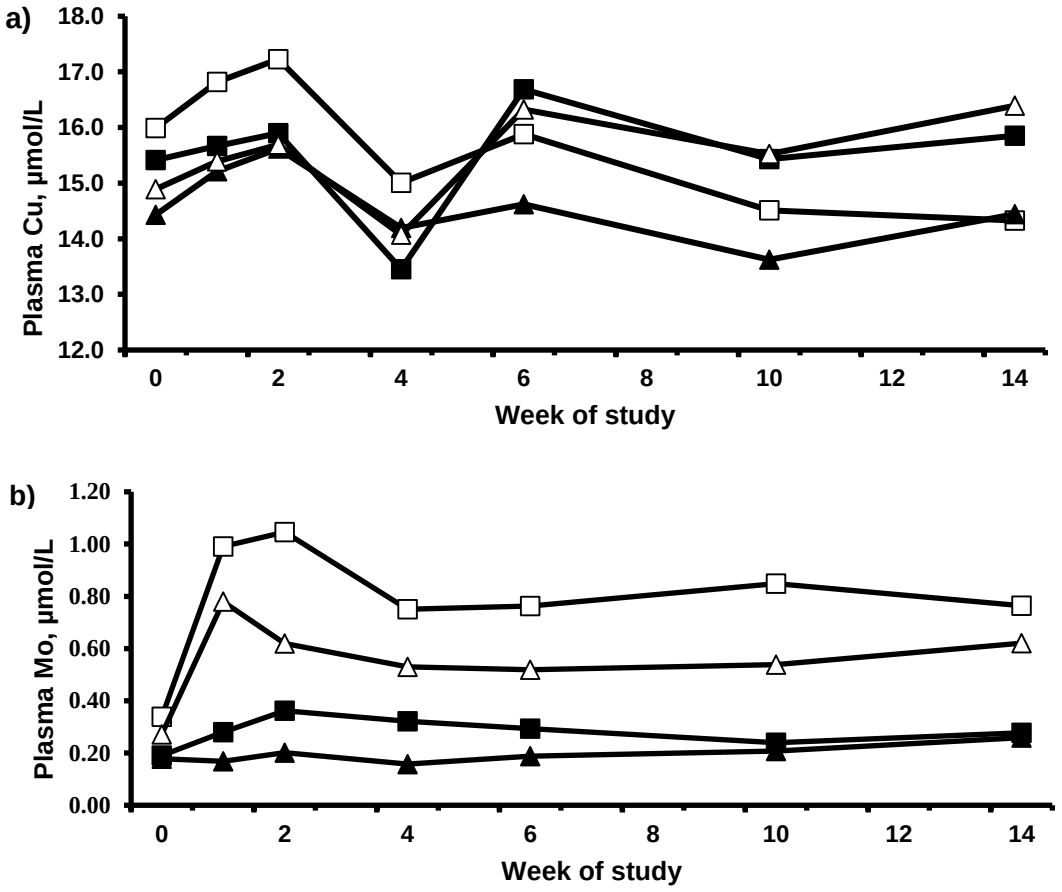

Figure 4.4. Plasma copper (a) and molybdenum (b) concentrations of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. For plasma copper; pooled SED = 1.07. Starch, P = 0.387; antagonist, P = 0.324; starch x antagonist, P = 0.454; time < 0.001; time x starch, P = 0.399; time x antagonist, P = 0.928; time x starch x antagonist, P = 0.003. For plasma molybdenum; pooled SED = 0.051. Starch, P = 0.004; antagonist, P < 0.001; starch x antagonist, P = 0.037; time, P < 0.001; time x starch, P < 0.001; time x antagonist, P = 0.006; time x starch x antagonist, P < 0.001.

4.3.3 Haematological profile

Cows fed additional S and Mo had 0.8, 0.04, and 0.60 $10^3/mm^3$ higher ($P < 0.05$) WBC, monocyte and neutrophil counts compared to cows those fed unsupplemented diets (Table 4.5; Figure 4.5). White blood cell and monocyte counts were also 0.71 and 0.03 $10^3/mm^3$ higher ($P < 0.05$) in cows fed the high compared to the low starch diets. There was also an interaction between dietary starch concentration and antagonist supplementation on monocyte counts, which increased when low starch diets were fed with additional S and Mo, but decreased when high starch diets were fed with additional S and Mo. Monocyte and neutrophil counts fluctuated ($P < 0.05$) throughout the duration of the study (Figure 4.5b,c). Red blood cell and eosinophil counts were unaffected ($P > 0.05$) by dietary starch inclusion or S and Mo supplementation. Cows fed the antagonist supplemented diets had 0.4 g/dL higher ($P < 0.05$) haemoglobin concentrations than those fed the unsupplemented diets. There was also a trend ($P < 0.1$) for lower haematocrit percentages in cows fed containing additional S and Mo.

Table 4.5. Haematological profile of dairy cows fed low (LS) or high starch (HS) diets without (-) or with (+) added S and Mo.

	Diet					P-value[1]		
Item[3]	LS-	LS+	HS-	HS+	SED	S	A	Int
WBC[2], $10^3/mm^3$	9.5	9.6	8.1	9.5	0.48	0.045	0.032	0.094
Mon No[2], $10^3/mm^3$	0.37	0.36	0.29	0.38	0.021	0.037	0.022	0.003
Neu No[2], $10^3/mm^3$	5.24	5.56	4.63	5.51	0.360	0.191	0.020	0.302
Lym No, $10^3/mm^3$	3.34	3.41	2.74	3.35	0.298	0.129	0.122	0.208
Eo No, $10^3/mm^3$	0.14	0.13	0.14	0.14	0.022	0.881	0.639	0.498
RBC, $10^6/mm^3$	6.30	6.58	6.46	6.53	0.178	0.693	0.164	0.423
HCT[2], %	32.0	33.0	31.8	32.9	0.048	0.802	0.064	0.984
Hb[2], g/dL	10.0	10.3	9.9	10.3	0.22	0.612	0.026	0.708

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.
[2] Wk 0 values used as a co-variate.
[3] White blood cells (WBC), monocyte numbers (Mon No), neutrophil numbers (Neu No), lymphocyte numbers (Lym No), eosinophil numbers (Eo No), red blood cell count (RBC), haematocrit percentage (HCT), and haemoglobin (Hb).

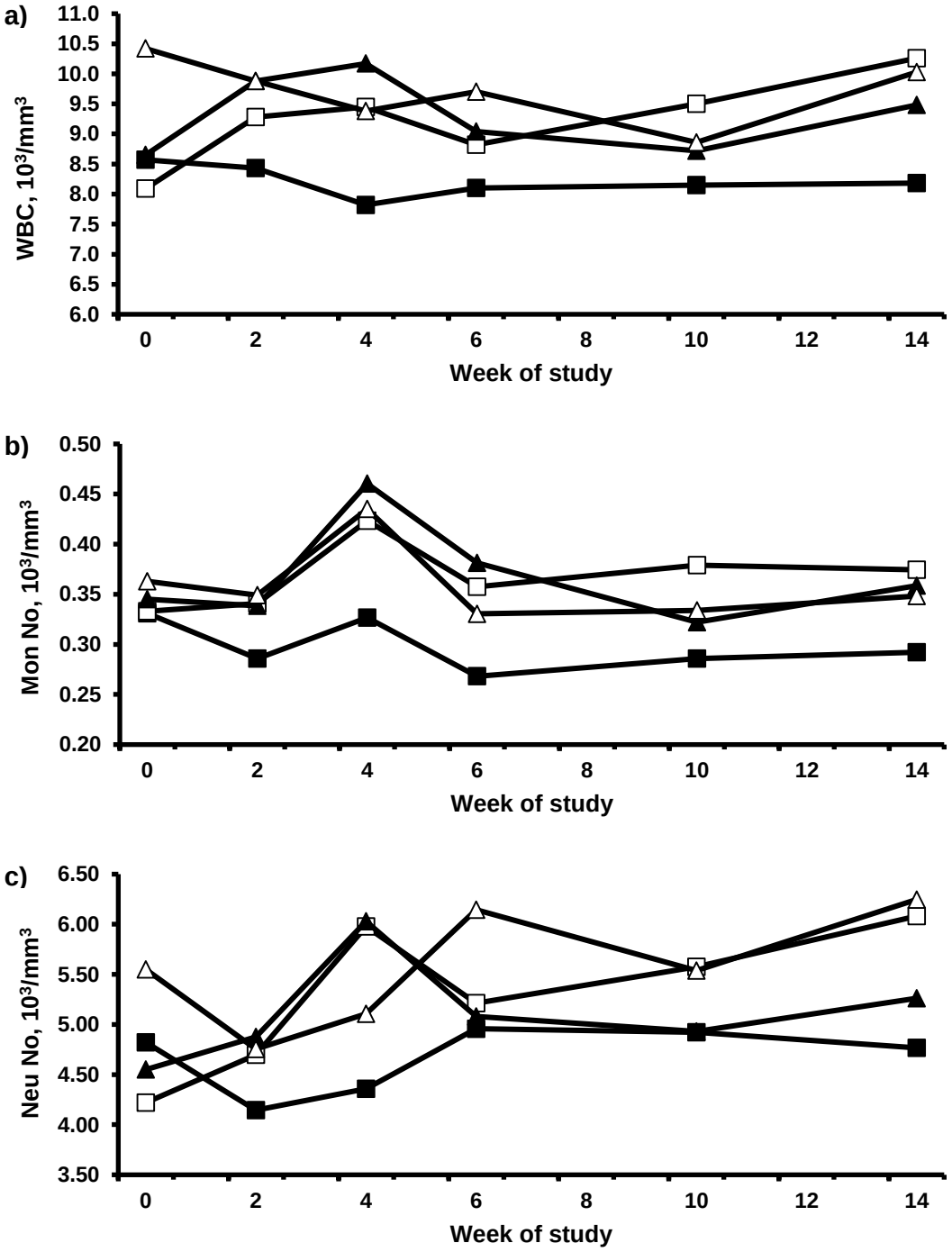

Figure 4.5. White blood cell count (WBC; a), monocyte number (Mon No; b), and neutrophil number (Neu No; c) of early lactation dairy cows fed low starch diets without (▲) or with (△) added S and Mo, or high starch diets fed without (■) or with (□) added S and Mo. For white blood cell count; pooled SED = 0.77. Starch, P = 0.045; antagonist, P = 0.032; starch x antagonist, P = 0.094; time, P = 0.219; time x starch, P = 0.416; time x antagonist, P = 0.656; time x starch x antagonist, P = 0.497. For monocyte number; pooled SED = 0.036. Starch, P = 0.037; antagonist, P = 0.022; starch x antagonist, P = 0.003; time, P < 0.001; time x starch, P = 0.210; time x antagonist, P = 0.832; time x starch x antagonist, P = 0.564. For neutrophil number; pooled SED = 0.656. Starch, P = 0.191; antagonist, P = 0.020; starch x antagonist, P = 0.302; time, P = 0.048; time x starch, P = 0.867; time x antagonist, P = 0.555; time x starch x antagonist, P = 0.123.

4.3.4 Hepatic mineral concentration

Cows fed the high starch diets had liver Cu concentrations at the end of the study (551 and 471 mg/kg DM for HS- and HS+) that were 57 mg/kg DM higher ($P < 0.05$) than cows fed the low starch diets (522 and 381 mg/kg DM for LS- and LS+ respectively; Table 4.6). There was also an effect of Cu antagonists on final hepatic Cu concentration which was 108 mg Cu/kg DM lower ($P < 0.001$) in cows fed the S and Mo supplemented compared to the unsupplemented diets. The consequence of these effects was a net gain in liver Cu concentrations across three of the dietary treatments, with the exception being cows fed the low starch diets containing added S and Mo (LS+) which had a net loss. There was however no interaction ($P > 0.05$) between starch concentration and Cu antagonists on the rate of change or final hepatic Cu concentration. There was no difference ($P > 0.05$) in initial hepatic Mo concentration between treatments, but hepatic Mo concentrations at the end of the study were higher ($P < 0.05$) in cows fed diets supplemented with S and Mo (LS+ and HS+). The was also an effect of dietary starch concentration on final hepatic Mo concentration, where cows fed the high starch diets had 3.71 mg/kg DM lower ($P < 0.05$) Mo concentrations at the end of the study compared to those fed the low starch diets. Finally, there was no effect ($P < 0.05$) of dietary treatment on hepatic Fe or Zn concentration, although cows fed the diets supplemented with S and Mo tended ($P < 0.1$) to have a net gain in Zn.

Table 4.6. Liver mineral concentrations in dairy cows fed low (LS) or high starch (HS) diets without (-) or with (+) added S and Mo.

	Diet					P-value[1]		
	LS-	LS+	HS-	HS+	SED	S	A	Int
Initial Cu, mg/kg DM	474	476	466	401	40.6	0.155	0.284	0.247
Final Cu[2], mg/kg DM	522	387	551	471	34.2	0.018	<0.001	0.262
Δ Cu[2], mg/kg DM/d	0.72	-0.65	1.02	0.20	0.349	0.019	<0.001	0.262
Initial Fe, mg/kg DM	278	241	291	307	29.9	0.071	0.632	0.222
Final Fe, mg/kg DM	285	255	271	273	48.9	0.972	0.686	0.648
Δ Fe, µg/kg DM/d	78	143	207	352	424.9	0.204	0.895	0.729
Initial Mo, mg/kg DM	3.52	3.58	3.83	3.60	0.323	0.467	0.713	0.529
Final Mo[2], mg/kg DM	3.76	4.03	3.58	3.83	0.127	0.049	0.008	0.869
Δ Mo[2], µg/kg DM/d	1.54	4.32	-0.22	2.25	1.296	0.049	0.008	0.867
Initial Zn, mg/kg DM	99.5	85.1	78.1	78.9	12.91	0.142	0.462	0.410
Final Zn, mg/kg DM	85.7	95.2	75.6	93.4	10.38	0.426	0.073	0.578
Δ Zn, mg/kg DM/d	-0.14	0.10	-0.03	0.15	0.161	0.487	0.076	0.757

[1] S= main effect of starch level, A = main effect of antagonists, Int = interaction between starch level and antagonists.
[2] Wk 0 values used as a covariate.

4.4 Discussion

4.4.1 Performance and Intake

This study is the first to determine Cu status and metabolism in early lactation high yielding dairy cows fed different concentrations of dietary starch. The mean dietary Cu concentration of 15 mg Cu/kg DM used in this study was 12.9 mg/kg DM lower than the mean concentration observed by Sinclair and Atkins (2015) in winter-fed early lactation dairy cow diets in the United Kingdom. It was however similar to the mean concentration of 18 mg Cu/kg DM reported by Castillo et al. (2013) on Californian dairy farms. The LS+ and HS+ diets were supplemented with dietary S and Mo at levels that were predicted to reduce Cu absorption and substantially alter Cu metabolism. Using the equations of Suttle and McLauchlin (1976), it was predicted the apparent Cu absorption coefficient of the LS+ and HS+ diets was 0.021, approximately 50% lower than the 0.042 predicted for the LS- and HS- diets respectively. The use of current equations however did not predict any effect of dietary starch concentration on Cu status or performance.

The primary challenge in dairy cow feeding is providing an energy dense ration in order to minimise the negative energy balance that the cow experiences during early lactation without compromising the ruminal ecosystem (Humer *et al.*, 2018; Zebeli *et al.*, 2008). This ecosystem can become compromised in certain instances, particularly where high dietary concentrations of grain-based high starch concentrates are supplied (Humer *et al.*, 2008), resulting in the rapid generation of short-chain fatty acids (SCFA; Aschenbach *et al.*, 2010). The rapid production of these molecules relative to that of buffer disrupts pH regulation within the rumen (Steele *et al.*, 2011), and consequential intermittent drops in pH of sufficient length and magnitude can cause subacute ruminal acidosis (SARA; Dirksen, 1985), a condition associated with depressed fiber digestion, intakes, yield, and milk composition (NRC, 2001). There is however much debate surrounding the ruminal pH changes that define SARA (Humer *et al.*, 2018), some studies use the duration per day below a threshold pH value (Krause *et al.*, 2003), whilst others identify SARA when the daily nadir in ruminal pH is below a critical threshold (Zebeli *et al.*, 2008), although estimates of this threshold nadir range from 5.6 to 6.0 (Keunen *et al.*, 2002; Krehbiel *et al.*, 1995). Zebeli *et al.* (2008) conducted a meta-analysis examining the effects of dietary fiber on ruminal pH and concluded that in order to minimise SARA, ruminal pH should not be below 5.8 for longer than 5.24 h/d, and mean ruminal pH should be no less than 6.16. The decreased mean reticular pH values in cows fed the high starch/low NDF diets observed in the current study supports the findings of Tafaj *et al.* (2007) who reported a strong positive correlation

(R^2 = 0.41) between rumen pH and dietary NDF content. Cows fed the high starch diets had a mean ruminal pH value of 6.23, and spent 1.5 h/d below pH 5.8, and were subsequently well above the threshold for SARA according to Zebeli *et al.* (2008). The primary reason for increasing dietary starch concentrations in this study was not to assess SARA risk, but to emulate the potential changes in ruminal pH when grass silage is substituted for maize silage in the diet (Sinclair *et al.*, 2017; Tayyab *et al.* 2018). This was indeed achieved with a similar reduction in rumen pH in the current study to that reported by Tayyab *et al.* (2018) when maize silage was substituted for grass silage in the diet of lactating Holstein-Friesian dairy cows.

Similar to other studies that have investigated the effects of S supplementation on intake (Spears et al., 2011; Drewnoski and Hansen, 2013), DMI in the current study was reduced by 1.8 kg/d in the diets supplemented with S and Mo. It is not possible to determine the effects of S or Mo in isolation in the current study, but the potential of S to reduce intake has been widely studied in North American beef cattle that have been exposed to high dietary S concentrations resulting from the dietary inclusion of ethanol co-products (Drewnoski *et al.*, 2014a). Spears *et al.* (2011) reported a decrease in DMI when dietary S inclusion exceeded 2 g/kg DM, in contrast, Richter *et al.* (2012) reported no effect of dietary S concentration on DMI at concentrations as high as 6 g/kg DM. Conflicting evidence regarding the effects of S on DMI is not restricted to dietary concentration alone, with Sinclair *et al.* (2017) reporting little effect of additional S and Mo on DMI when dairy cows were fed maize silage-based diets, but observed a 2.1 kg DM/d decrease when a grass silage-based diet was fed. Sinclair *et al.* (2013) also reported a decrease in DMI when Cu-proteinate was substituted for $CuSO_4$ in diets containing additional S and Mo. In the rumen, inorganic sulfates and/or S-containing amino acids are converted to hydrogen sulfide (H_2S) by sulfur reducing bacteria, which is subsequently excreted into the ruminal fluid (Bradley *et al.*, 2011). Some of this H_2S disassociates to form bisulfide (HS^-) in the liquid fraction whilst the remainder migrates to the gas cap of the rumen (Schoonmaker and Beitz, 2012). This reaction is thought to be pH-dependent with a greater disassociation of H_2S at higher pH values. For example, at a pH of 7.0 and a pKa of 7.04, approximately 50% of H_2S will disassociate to form HS^- compared to a 5% disassociation rate at a pH of 5.5 (Drewnoski *et al.*, 2014a). In the current study, it is estimated using mean ruminal pH vales that cows fed the low starch diets would have had a 5% greater dissociation of H_2S to form HS^- in the liquid fraction of the rumen throughout the day (Schoonmaker and Beitz, 2012). It is speculated that ruminal H_2S that makes its way to the gas cap is eructated and inhaled, eventually reaching the brain where it can cause polioencephalomalacia (PEM) and associated necrosis of the grey matter (Dougherty and Cook, 1962; Gould *et al.*, 1997). Dry

matter intake is then reduced either through direct effects on the brain and/or ruminal discomfort (Richter *et al.*, 2012). This provides a possible explanation for the greater decrease in DMI in cows fed the high starch diets in this study, where lower rumen pH values resulted in a greater proportion of ruminal S being present as H_2S (Drewnoski *et al.*, 2014a; Gould *et al.*, 1987; Richter *et al.*, 2012). It is indeed hypothesised that ruminal pH is the major reason for the differences in sulfur tolerance between forage and concentrate fed cattle (Drewnoski *et al.*, 2012). Unfortunately, it was not possible to measure rumen H_2S production in the current study due to its invasive time-consuming nature, and studies that have, tend to focus solely on this variable (Drewnoski and Hansen, 2013; Drewnoski *et al.*, 2014b). Measuring ruminal H_2S in cows which are not cannulated involves the insertion of a 16-gauge 10.2 mm long needle into the ruminal gas cap through the left paralumbar fossa, elastomer tubing connected to this needle can then be used to facilitate ruminal gas flow through an H_2S detector (Drewnoski *et al.*, 2012). Given that a possible effect of diet type on ruminal H_2S was only one of a number of hypothetical outcomes in this study, and cows were already experiencing the stress associated with two liver biopsies, it was not considered ethically appropriate to measure hydrogen sulfide.

Evidence relating the effects of dietary starch concentration on milk fat production is conflicting, with several authors having reported a decrease in milk fat yield associated with increasing dietary starch concentration (Sutton, 1989; Poore *et al.*, 1993), whilst other others have reported no effect (Chanjula *et al.*, 2004; Jurjanz *et al.*, 1998). These conflicting findings may be explained by a number of factors including starch source, degradation rate, and quantity supplied (Gómez *et al.*, 2016). The dietary starch supply of approximately 4.5 and 5.0 kg DM/d for cows fed the HS- and HS+ diets respectively is well below the 6.8 kg of DM /d hypothesised by Casper *et al.* (1990) to cause modified volatile fatty acid synthesis in the rumen and reduced milk fat (Reynolds *et al.*, 1997). The increase in milk protein concentration and yield as dietary starch concentration increases in this study is consistent with the findings of other studies (Carmo *et al.*, 2014; Hills *et al.*, 2015), who reported a linear increase in milk protein yield up to an optimal 250 g/kg DM dietary concentration (Hills *et al.*, 2015). Increasing dietary starch concentrations have been shown to increase fermentable metabolisable energy resulting in greater microbial protein synthesis (Oba and Allen, 2003). Diets which maximise microbial protein synthesis tend to have an increased digestibility and an amino acid profile which is closer to the cow's requirements (O'Connor *et al.*, 1993), and as a result have the potential to increase milk protein yield (Carmo *et al.*, 2014). Neither rumen volatile fatty acid production nor microbial efficiency were monitored in the current study, and so definitive explanations for the effects of starch or lack thereof

upon milk yield and composition are difficult to ascertain (Beckman and Weiss, 2005; Oba and Allen, 2003).

Cows fed additional S and Mo had a lower ME intake, but a similar ME output compared to cows fed the unsupplemented diets, this resulted in an increased ME balance in cows fed additional S and Mo. This was surprising given that there was no evidence of the additional tissue mobilisation that would have been required to provide the energy needed to sustain milk production in cows fed the antagonist supplemented diets (Roche *et al.*, 2009). There may however be an explanation relating to the increased S content of the antagonist supplemented diets (Drewnoski *et al.*, 2014a). Uwituze *et al.* (2011) reported a decreased DMI in steers when dietary S concentration was increased from 4.2 to 6.5 g S/kg DM, despite an increase in dry and organic matter digestibilities. It was hypothesised that the additional H_2S produced in the 6.5 g S/kg DM diet decreased gastrointestinal tract motility, resulting in a greater rumen retention time and subsequently digestibility (Uwituze *et al.*, 2011). Evidence of decreased rumen motility as a result of increasing dietary S concentrations originates from Delfiol *et al.* (2013), who reported that sheep fed 9 g S/kg DM had less frequent rumen movements than those fed 2 g S/kg DM. The dietary S concentrations reported in these studies however are well above the 3 g S/kg DM in the antagonist supplemented diets reported in the current study. These effects have however been reported at much lower dietary S concentrations, Thompson *et al.* (1972) reported decreased DMI but no effect on steer ADG when dietary S concentrations were increased from 1.2 to 3.7 g S/kg DM. This increased efficiency as a result of decreased rumen motility may account for the lack of tissue mobilisation despite a greater negative energy balance in cows fed the antagonist supplemented diets in the current study (Drewnoski *et al.*, 2014a; Roche *et al.*, 2009), although digestibility was not monitored.

4.4.2 Plasma Mineral Profile, Cu-mediated enzymes and acute phase proteins

In accordance with other studies that have examined the effects of Cu antagonists on Cu status (Sinclair *et al.*, 2017; Scaletti and Harmon, 2012), there was no effect of dietary treatment on plasma Cu concentrations which were all well in excess of the 9 µmol/L considered to be adequate (Laven and Livesey, 2005). This finding supports previous observations that plasma Cu is a poor indicator of Cu status unless the animal is experiencing either very high or low hepatic Cu reserves (Dias *et al.*, 2013). Similar to other studies that have investigated the effects of S and Mo supplementation (Sinclair *et al.*, 2013; Sinclair *et al.*, 2017), elevated plasma Mo was observed in cows fed the Cu antagonist

supplemented diets. There was also an interaction between dietary starch concentration and Cu antagonists with respect to plasma Mo concentration, which increased by an additional 0.2 µmol/l when cows were fed the HS+ compared to the LS+ diets. Dietary sulfides are thought to combine with molybdate in the rumen to form thiomolybdates, that subsequently form a complex with Cu preventing its absorption (Suttle, 1991). This reaction is both pH-dependent and reversible, where molybdate reacts with HS^- in a stepwise manner commencing with the formation of monothiomolybdate through to the di-, tri-, and tetrathiomolybdate isomers sequentially (Gould and Kendall, 2011). These different species are suggested to have differing consequences for Cu absorption and metabolism (Suttle, 1991). Tri- and tetrathiomolybdates are thought to irreversibly bind Cu to high molecular weight proteins rendering it unavailable for absorption (Suttle and Field, 1983), whereas the lower isomers such as monothiomolybdate are thought to be broken down by the acidity the abomasum, freeing any complexed Cu and molybdate for absorption (Price *et al.*, 1987; Suttle, 1991). Clarke and Laurie (1980) identified increased rates of tetrathiomolybdate formation *in vitro* at lower ruminal pH values, however they also noted that rumen pH may affect S supply to the reaction *in vivo* with possible consequences for thiomolybdate speciation (Clarke and Laurie, 1980). If therefore a lower proportion of S was present in the liquid phase as HS^- because of a reduced ruminal pH in cows fed the high starch diets, the proportion of tri- and tetrathiomolybdate formed may also be reduced due to decreased S supply to the reaction (Drewnoski *et al.*, 2014a; Gould and Kendall, 2011). The greater increase in plasma Mo in cows fed the high starch diets may therefore possible be explained by a greater proportion of mono- and dithiomolybdates in cows fed the high starch diets, which are then subsequently broken down in the abomasum freeing the molybdate for absorption into the bloodstream (Clarke and Laurie, 1980; Price *et al.*, 1987).

Evidence is conflicting regarding the effect of feeding additional S and Mo on Cp activity (Sinclair *et al.*, 2013; Ward and Spears, 1997). Ward and Spears (1997) reported decreased Cp activity in growing and finishing steers receiving a diet containing additional Mo. In contrast, Sinclair *et al.* (2013; 2017) reported no effect on Cp activity as a result of the dietary addition of S and Mo. Sinclair *et al.* (2017) did however report an effect of forage type on Cp activity which decreased by 2.3 mg/dL when grass silage was substituted for maize silage in the diet. In the current study, Cp activity was found to increase in cows fed the high starch diets. There are a number of possible explanations for this increase. Firstly, Cp can act as a minor acute-phase protein (Kaya *et al.*, 2016), and so the decreased rumen pH in cows fed the high starch diets may have resulted in an acute phase response triggering increased Cp synthesis (Cannizzo *et al.*, 2012). In this case however an increase in the major acute-phase protein haptoglobin was likely in combination with a decrease in

circulating plasma Zn and Fe (Plaizier *et al.*, 2009), and/or an increase in plasma Cu (Sattar *et al.*, 1997). Neither an increase in serum Hp or an effect of dietary starch concentration on plasma Zn, Fe, and/or Cu was noted in this study. Another potential explanation lies in the lower Cu status of cows fed on the low starch diets, as Cp activity has been shown to decrease along with Cu status in cases of Cu deficiency (Blakey and Hamilton, 1985). The rate of Cp synthesis however is thought to be relatively constant provided hepatic Cu storage is not limiting (Hellman and Gitlin, 2002). In the current study, hepatic Cu concentrations across all four dietary treatments were well above the 19 mg Cu/kg DM limit suggested to pose a risk of deficiency symptoms (Laven and Livesey, 2005), and it is therefore unlikely that hepatic Cu storage was a limiting factor during Cp synthesis (Blakey and Hamilton, 1985).

Another possible explanation for altered Cp activity in this study relates to the aforementioned potential increase in trithiomolybdate formation in cows fed the low starch diets as a result of increased S supply to this reaction at higher rumen pH values (Clarke and Laurie, 1980; Drewnoski *et al.*, 2014a). Trithiomolybdates have been known to pass into the bloodstream (Mason et al., 1982), where they complex albumin and have the potential to inhibit Cp synthesis and/or activity (Keller and Mason, 1986). Thiomolybdate absorption however is a controversial subject area, Suttle (2010) theorised that absorption is unlikely unless the Cu: Mo ratio is below 1:1, which is well below the mean value of 3.3:1 fed in the LS+ and HS+ diets, although Suttle (2010) also noted wide ranges in this ratio as a result of factors such as feed composition and dietary sulfur concentrations. If indeed, a greater proportion of trithiomolybdate was produced in the low starch diets as a result of increased S supply to the thiomolybdate reaction (Gould and Kendall, 2011), Cp activity may potentially have been inhibited in these diets as a result of trithiomolybdate absorption (Mason *et al.*, 1982). Thiomolybdate speciation in the rumen was not however monitored in the current study. Similar to other authors who have examined the effects of elevated dietary S and Mo on Cu-dependent enzymes (Sinclair *et al.*, 2013; 2017; Ward *et al.*, 1993), the activity of SOD, an enzyme involved in the bodies defence against free radicals (Suttle, 2010), was unaffected by dietary treatment. This is not unexpected however as SOD is usually one of the last enzymes to reflect a change in Cu status (Paynter, 1987), the relatively long lifespan of erythrocytes in relation to the current study length in this instance means that observed SOD effects were unlikely before day 98 of the study (Suttle and McMurray, 1983; Ward *et al.*, 1993).

4.4.3 Haematological Profile

There exists a variety of potential explanations why cows experienced leukocytosis when fed either low starch diets or additional S and Mo (Roland *et al.*, 2014). Changes in rumen pH, and in particular the onset of subacute ruminal acidosis, have previously been associated with an acute phase response causing increased white blood cell, monocyte, neutrophil and eosinophil counts (Ceroni *et al.*, 2012; Rodríguez-Lecompte *et al.,* 2014). This however seems an unlikely explanation for the elevated WBC counts in cows fed the low starch diets given that the mean reticular pH of these animals was higher than those fed the high starch diets. Arthington *et al.* (1996) reported neutropenia in Angus x Hereford heifers receiving a diet with a Cu: Mo ratio of 1: 2.5 despite liver Cu concentrations of 49 mg/ kg DM that were well in excess of the 19 mg/kg DM threshold hypothesised to place cattle at risk of Cu deficiency (Laven and Livesey, 2005). Biological mechanisms for this neutropenia evaded Arthington *et al.* in (1996), whether it was the influence of the dietary Mo (Boyne and Arthur, 1981), the thiomolybdates that would have been formed as a result of the S present in the diet (Suttle, 1991), or indeed the decreasing Cu status of the heifers over the duration of the study (Minatel and Carfignini, 2000). Indeed, any of these factors in isolation or combination could have caused the neutropenia observed in cows fed the antagonist supplemented diets in the current study.

Possible explanations for the observed leukocytosis in cows fed the S and Mo supplemented diets in this study may be more straightforward. It is thought that necrosis of the grey matter occurs in cattle when S enters the brain during S induced PEM (Truong *et al.* 2006). The resulting deceased neurons and their processes are then phagocytosed predominantly by monocytes which originate from the bloodstream (Paula *et al.*, 2018). Both monocytosis and neutrophilia have been associated with tissue necrosis in bovines (Roland *et al.*, 2014), and so leukocytosis may be a secondary effect of the additional H_2S produced in cows fed the S and Mo supplemented diets (Drewnoski *et al.*, 2014; Roland *et al.*, 2014). Symptoms associated with PEM include oxidative-antioxidative imbalance, blood vessel dysfunction, and hypoxia of the cerebral cortex (Lutnicki *et al.*, 2014). Tissue hypoxia may account for the increased haemoglobin (Hb) concentrations in cows fed the diets containing additional S and Mo (Roland *et al.*, 2014). Secondary polycythaemia is known to result from hypoxia, where erythropoietin secretion from the kidneys is increased stimulating erythropoiesis in an attempt by the cardiovascular system to increase the oxygen carrying capacity of the blood (Jones and Allison, 2007; Manalo *et al.*, 2008). The subsequent increased erythrocyte count results in increased whole blood Hb concentrations (Brokus, 2011).

4.4.4 Hepatic Mineral Concentration

The liver is regarded as the main Cu storage organ (Laven and Livesey, 2005), and the first biochemical change to occur under Cu deprivation is a decrease in hepatic Cu concentration (Suttle, 2010). In this study, final hepatic Cu concentrations were 522 mg Cu/kg DM and 429 mg Cu/kg DM for cows fed unsupplemented or additional S and Mo respectively. The mean final hepatic Cu concentration of animals that did not receive additional S and Mo was above the upper threshold of 508 mg/kg of DM suggested by Livesey *et al.* (2002) to pose a risk of cows succumbing to clinical Cu toxicity. It is important to note a positive hepatic Cu balance across 3 treatments (LS-, HS- and HS+) despite dietary Cu concentrations in this study being comparable to the nutritional recommendations of NRC (2001). Current prediction equations do not account for dietary starch concentration and/or rumen pH modulation (Suttle and McLauchlin, 1976), and subsequently would not have predicted the increased hepatic Cu retention in cows fed the high starch diets in the current study (Suttle, 2010; Suttle and McLauchlin, 1976). The current study however is not the first to report an influence of dietary factors other than antagonists on apparent Cu availability (Sinclair *et al.*, 2017). Sinclair *et al.* (2017) reported similar effects where there was a greater decrease in hepatic Cu retention when cows were fed additional S and Mo in a grass silage- as opposed to a maize silage-based diet. There are two potential explanations that may explain an effect of dietary starch concentration on Cu absorption. Firstly, if a greater proportion of S is being eructated as H_2S in the high starch diets (Drewnoski *et al.*, 2014a), then the amount of S available for thiomolybdate formation decreases, which decreases the quantity of Cu locked in an insoluble thiomolybdate complex (Suttle, 1991). Secondly, another potential consequence of reduced S supply during Cu-S-Mo complex formation may be an increased proportion of lower thiomolybdate isomers (Mono- and dithiomolybdates) in cows fed the high starch diets (Clarke and Laurie, 1980). These lower isomers may subsequently break down in the abomasum freeing complexed Cu for absorption (Price *et al.*, 1987). Both instances would lead to an increase in apparent Cu availability (Suttle, 1991; Suttle and Field, 1983). Sinclair *et al.* (2017) did not monitor ruminal pH, although Tayyab *et al.* (2018) reported a decrease in reticular pH when grass silage was substituted for maize silage in the diet, which may explain the decreased hepatic Cu retention reported by Sinclair *et al.* (2017) when maize silage was substituted for grass silage.

In cows fed the LS- or HS- diets, feeding 15 mg Cu/kg DM resulted in a rapid increase in hepatic Cu, whilst cows fed HS+ also experienced a gradual gain. In contrast, at the rate of hepatic Cu decline in cows receiving LS-, hepatic Cu concentrations would eventually reach

the 19 mg Cu/ kg DM considered to pose a deficiency risk over a period of 566 days (Laven and Livesey, 2005). Given that feeding the same concentration of dietary Cu results in such large differences in hepatic Cu status, dietary starch concentration and/or rumen pH as well as dietary S and Mo be taken into consideration when calculating adequate Cu supplementation rates for dairy cows.

Molybdate that has been absorbed is normally stored in tissues such as the kidneys and liver as molybdoprotein where it binds to various enzymes of the mitochondria and cytosol (Johnston, 1997). Similar to other studies, an increase in hepatic Mo retention was observed as a result of S and Mo supplementation (Sinclair *et al.* 2013; 2017). Surprisingly however, hepatic Mo concentrations were lower in cows fed the high starch diets despite higher concentrations of circulating Mo in the bloodstream. Following intravenous administration, trithiomolybdate has been traced to the liver where it arrived intact (Wang *et al.*, 1987). Increased proportions of lower thiomolybdate isomers such as mono- and dithiomolybdate in cows fed the high starch diets may therefore explain the decreased hepatic Mo retention in the high starch diets despite elevated plasma Mo concentrations (Suttle and McMurray, 1983; Wang *et al.*, 1987). No effect of dietary treatment on hepatic Fe or Zn was reported in this study, with the liver generally not regarded as a major storage organ for either of these minerals. (Suttle, 2010).

4.5 Conclusion

Feeding lactating Holstein-Friesian dairy cows Cu at a dietary concentration of 15 mg Cu/kg DM in the absence of significant concentrations of S and Mo results in hepatic Cu accumulation. This demonstrates that this level of feeding is above the animal's requirements and supports current nutritional recommendations of 10-15 mg Cu/kg DM in lactating cow diets (NRC, 2001; Sinclair and Mackenzie, 2013). In contrast, feeding 15 mg Cu/kg DM in a diet with high S and Mo concentrations will cause hepatic Cu depletion in low starch diets, but has a much smaller effect in high starch diets. These differences in apparent Cu availability as a result of varying dietary starch concentration may be related to subsequent rumen pH modulation. Reasons for these differences in Cu absorption and metabolism as a result of dietary starch concentration and/or rumen pH are unclear and require further investigation. There is however a need to take these factors into account when calculating appropriate Cu supplementation strategies for lactating dairy cows.

CHAPTER 5: Impact of Cu supplementation level during the rearing period on Holstein-Friesian heifer performance, fertility, health, and indicators of Cu status.

5.1 Introduction

Copper (Cu) is an essential trace element and its supply has important implications for the performance, health, and fertility of lactating dairy cattle (Suttle, 2010). Clinical Cu deficiency presents with symptoms such as impaired growth and anaemia (McDowell, 1985; Suttle *et al.*, 1987), whilst several authors have associated appropriate Cu supplementation with increased fertility (Mackenzie *et al.*, 2001; Black and French, 2004). Recently there has been evidence of over-supplementation of the UK's winter-fed dairy cows (Sinclair and Atkins, 2015), with 32 out of 50 herds surveyed in Central and Northern England having a dietary Cu concentration in excess of the industry guideline maximum of 20 mg Cu/kg DM (ACAF, 2010). Indeed, six of the farms surveyed had a dietary Cu concentration in excess of the 40 mg/kg DM legal limit set by EU regulation 1831/2003 on animal feed additives (Sinclair and Atkins, 2015). It has also been reported that approximately 38% of the UK's Holstein-Friesian dairy cull cows had liver Cu concentrations exceeding the 508 mg Cu/kg DM threshold considered to pose a risk of clinical Cu toxicity (Kendall *et al.*, 2015; Livesey *et al.*, 2002). Despite the evidence of over-supplementation, a considerable number of British dairy herds continue to supply Cu at excessive levels (Jacklin, 2016). It has been suggested that this over-supplementation may be related to the perception that in the absence of clinical Cu toxicity, there is no detrimental effect of dietary Cu inclusion on dairy cow health, performance, or fertility (Kendall *et al.*, 2015). There is growing evidence however to suggest harmful sub-clinical consequences of over-supplementation as demonstrated by decreased live weight gain, impaired rumen function, and increased calf mortality (Arthington, 2005; Engle and Speers, 2000; Hunter *et al.*, 2013). Studies examining the effects of dietary Cu concentration have tended to be short term in duration, focused primarily on beef cattle, and have neglected aspects relating to health (Engle and Speers, 2000; Engle *et al.*, 2001; Ward *et al.*, 1993). There is however anecdotal evidence of the negative effects of Cu over-supplementation from practising vets who have reported a lowered immune status in dairy cattle as a result of elevated hepatic Cu concentrations (Howie, 2017). The literature also suggests that high dietary Cu concentrations can result in liver degeneration with the release of hepatic enzymes and a reduction in liver function that may impact upon health, fertility and performance (Laven and Livesey, 2005; Suttle, 2010). There is subsequently a need to determine the long-term effects of high dietary Cu inclusion levels upon replacement heifer health, performance and fertility.

5.2 Materials and Methods

5.2.1 Animals, treatments, housing and management

This study was conducted at Harper Adams University, Newport, Shropshire, UK, from September 2016 to September 2018. All of the procedures involving animals received local ethical approval and were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 (Amended Regulations 2013). Eighty Holstein-Friesian heifer calves that were 126 days of age (SE ± 1.9; 4.1 months of age; SE ± 0.1) and weighing 137 kg (SE ± 2.4) at the beginning of the study, were paired based on their live-weight, body condition score, and predicted transmitting ability (PTA; Swanson, 1991) for milk, fat and protein yield, and randomly allocated to one of two treatments. Each pair consisted of a Control (C) and a High (H) level of Cu supplementation. The control diet contained approximately 15 mg Cu/kg DM and was predicted to meet requirements (ARC, 1980; NRC, 2001; Table 2.4). Animals on the high treatment received the basal ration which was supplemented with an additional 15 mg Cu/kg DM to give a total dietary concentration of approximately 30 mg Cu/kg DM. This higher concentration was selected to be similar to the mean dietary Cu concentration of 28 mg/kg DM recorded on UK dairy farms by Sinclair and Atkins (2015). Heifers began to transition out of the growing phase (Chapter 5) and into the lactation study (Chapter 6) six weeks prior to their predicted calving date (23 months of age onwards), and as such results for the growing section of this study are presented up to 22 months of age.

Heifers were reared by the Harper Adams University Farm under commercial management conditions. Both animals within each pair received the same management and environmental conditions for the duration of the study, were kept in the same pen/paddock for the same period of time, and received the same basal diet. Within a pair, animals on H received two intra-ruminal copper oxide boluses (Agrimin Ltd, Kirmington, UK), which were formulated to supply approximately 15 mg Cu/kg DM based on predicted live-weight gain and DM intake throughout the growing phase (Hoffman *et al.*, 2008; Section 5.2.3). Each pair of boluses provided a 6-month Cu supply, with new boluses being administered thereafter. Animals on H recieved a pair of boluses containing 4.95 g, 7.64 g, and 16.55 g of CuO needles, supplying approximately 55, 73, and 160 mg of Cu/day, at 4, 10, and 16 months of age respectively.

Diet formulation was designed to achieve an average live-weight gain of 0.8 kg/d throughout the study period (AFRC, 1993). Prior to commencing the study all calves were fed a pelleted commercial calf grower (ForFarmers Ltd, Bury Saint Edmunds, UK) *ad-libitum* that contained wheat feed, palm kernel meal, sunflower seed meal, barley, sugar cane molasses, field beans, soya bean meal, vegetable fat, and minerals, which had a declared total macro mineral (g/kg DM) content of: 19.2 Ca, 7.6 P, 3.5 Mg, and 4.0 of Na, and a declared quantity of additional trace elements (mg/kg DM) of 30.0 Cu, 0.7 Se, 142.9 Zn, 9.9 Mn, 2.4 I, and 0.5 of Co. Upon commencing the study heifers recieved a total mixed ration (TMR; Table 5.1) *ad-libitum*, which was initially supplemented with 1.9 kg DM/d of the commercial calf grower. The quantity of grower fed was reduced under the commercial management of the farm, and subsequently removed at 7 months of age. Replacement heifers grazed swards composed predominantly of perennial rye grass during the summer months under a set-stock grazing regime, and were supplemented with the commercial calf grower at grass during their first grazing season in order to maintain a live weight gain of approximately 0.8 kg/d, and to provide a basal Cu concentration of approximately 15 mg/kg DM. During the second grazing season heifers recieved up to 0.8 kg DM/d of mineralised wheat whilst grazing to maintain a basal dietary Cu concentration of approximately 15 mg/ kg DM (Table 5.2). Heifers had continual access to fresh water containing mineral concentrations of S, Cu, Fe, and Mo of 18.8 mg/L, 3.6 µg/L, 1.9 µg/L, and 0.2 µg/L respectively.

Table 5.1. Composition of the total mixed ration fed to replacement dairy heifers when house between 4 and 22 months of age.

	Total Mixed Ration
Item, g/kg DM	
Grass silage	306
Maize silage	134
Chopped wheat straw	241
Spey syrup[1]	58
Soy hulls	100
Rapeseed meal	53
Wheat distillers dark grains	52
Soy bean meal[2]	22
Palm kernel meal	15
Molasses	4
Limestone flour	4
Mins/Vits[3]	11
Total	1000

[1] Pot ale syrup, KW Alternative Feeds, Andover, UK.
[2] Rumen protected soya bean meal, KW Alternative Feeds, UK.
[3] Mineral/vitamin premix, KW Alternative Feeds, Andover, UK. Major minerals (g/kg): Ca 220, P 30, Mg 60, Na 80; Trace minerals (mg/kg): Cu 0, Se 20, Zn 6,000, Mn 4,000, I 300, Co 80; vitamins (iu/kg) were: retinol 800,000, cholecalciferol 250,000, and all *rac* α-tocopherol acetate 2,000.

Table 5.2. Composition of the mineralised wheat fed to replacement dairy heifers during the second grazing season.

	Mineralised wheat
Item, g/kg DM	
Rolled wheat	803
Proflo syrup[1]	77
Mins/Vits[2]	120
Total	1000

[1] Wheat Vinasses, KW Alternative Feeds, Andover, UK. 12.5 mg of Cu/kg DM declared. Declared concentrations of S and Mo are not available.
[2] Mineral/vitamin premix, KW Alternative Feeds, Andover, UK. Major minerals (g/kg): Ca 230, P 40, Mg 50, Na 80; Trace minerals (mg/kg): Cu 1,000, Se 30, Zn 6,000, Mn 4,000, I 400, Co 75; vitamins (iu/kg) were: retinol 400,000, cholecalciferol 80,000, and all *rac* α-tocopherol acetate 2,000.

Table 5.3. Chemical analysis of the total mixed ration (TMR), grazed perennial ryegrass, pelleted concentrate, mineralised wheat and calculated mean diet fed to replacement dairy heifers from 4 to 22 months of age.

	Feed type				
	TMR	Perennial ryegrass[1]	Concentrate	Mineralised wheat	Mean diet composition[2]
Chemical analysis					
DM, g/kg	496	223	863	797	491
Crude protein, g/kg DM	152	198	195	169	163
Ash, g/kg DM	84	97	95	118	88
Organic matter, g/kg DM	916	903	905	882	912
NDF, g/kg DM	514	502	341	105	497
ADF, g/kg DM	324	281	170	39	304
Ether extract, g/kg DM	19	21	38	9	21
Starch, g/kg DM	77	--	181	542[3]	78
WSC, g/kg DM	38	161	75	26[3]	60
Ca, g/kg DM	7.99	4.73	18.12	28.14	9.19
P, g/kg DM	4.51	3.60	8.21	5.93	4.61
Mg, g/kg DM	2.36	1.79	3.72	4.39	2.41
S, g/kg DM	2.22	2.42	2.76	2.53	2.29
Cu, mg/kg DM	14.6	7.8	35.9	151.9	16.2[4]
Mo, mg/kg DM	1.6	3.3	0.9	0.6	1.6
Zn, mg/kg DM	65.0	42.2	170.2	808.6	85.3
Fe, mg/kg DM	335	134	240	341	285
Mn, mg/kg DM	83.2	58.7	102.6	419.5	87.7

[1] Set-stocked grazing, mean number of days at grass: 1st grazing season = 93 days, 2nd grazing season = 27 days, mean grass cover of 2684 kg DM/ha.

[2] Predicted contribution of each feed type to total DMI throughout the study (expressed as a percentage of total DMI): 76% TMR, 15% PRG, 9% concentrate, and 1% mineralised wheat.

[3] Starch and WSC content predicted using standard values: wheat (MAFF, 1982), proflo syrup and minerals/vitamins (KW Alternative Feeds, 2019).

[4] Heifers on treatment H were supplemented with 15.6 mg Cu/kg DM to provide a total dietary concentration of 31.8 mg/kg DM.

5.2.2 Experimental routine

Heifers were weighed, condition scored (Ferguson *et al.*, 1994) and wither height measured at the beginning of the study, and on a fortnightly basis thereafter. Wither height was determined through the use of a height measuring stick in a procedure adapted from Jeffery and Berg (1972). Oestrus detection was undertaken from 5 months of age according to Van Erdenburg *et al.* (2002). Heifers were observed by the same person for 30-minute periods at approximately 0700 h in the morning and 1900 h in the evening. Oestrus behaviour was recorded using the behavioural scoring system described by Van Erdenburg *et al.* (1996; Table 5.4). The assigned number of points was recorded each time the corresponding oestrus behaviour was observed, and heifers were considered to be in oestrus when the sum of the points exceeded 50 at two consecutive observation sessions (Van Erdenburg *et al.*, 2002). Oestrus detection continued throughout the insemination period until pregnancy confirmation. Heifers were served according to the Harper Adams University standard artificial insemination (AI) protocol from 13.5 months of age until ultrasound pregnancy confirmation. First services were by artificial insemination with either sexed (Holstein) or conventional (non-sexed; Holstein/ Aberdeen Angus) semen. Second services were also by artificial insemination using conventional (non-sexed) Holstein or Aberdeen Angus semen, the use of semen from different bulls was balanced across treatments where possible. Third and subsequent services were conducted through the use of a Hereford stock bull. If, however a heifer reached 18 months of age without conceiving, service was by the Hereford stock bull regardless of service number, and if pregnancy confirmation was not achieved by 22 months of age, the animal was subsequently culled. The birth weights of calves born to these heifers was recorded within 6 hours postpartum. Fresh samples of concentrate, TMR, grass, and mineralised wheat were collected on a fortnightly basis and stored at -20 ^{o}C prior to subsequent analysis.

Blood samples were collected via jugular venepuncture at 1100 h at the beginning of the study and bi-monthly thereafter into vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK) containing lithium heparin (to determine aspartate aminotransferase; AST), silica gel (to determine ceruloplasmin and glutamate dehydrogenase; Cp and GLDH), dipotassium ethylenediaminetetraacetic acid (K_2EDTA; to determine the haematological profile and superoxide dismutase activity; SOD), and potassium ethylenediaminetetraacetic acid (K_3EDTA; to determine plasma mineral concentration and gamma-glutamyl transferase; GGT). Liver biopsy samples were collected at 7, 13 months of age, and 6 weeks prior to predicted calving via the 11th intercostal space as described by Davies and Jebbet (1981). Biopsy samples were immediately snap frozen in liquid nitrogen and stored

at -80 °C prior to subsequent analyses. The humoral immune response of the heifers was tested according to Mackenzie *et al.* (1997a). Heifers were immunised at 1100 h subcutaneously over the 5th rib on the right side using 1 mL of 2 mg/mL ovalbumin (Sigma Aldrich, Dorset, UK) precipitated on alum at 17 months of age (Pollock *et al.*, 1991; 24 heifers, 12 per treatment). Blood samples were collected at 1100 h in vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK) containing silica gel at day 0 (pre-immunisation), and again on days 7, 14, 21, 28, and 63 post-immunisation, with serum being stored at -20 °C prior to subsequent analysis (Mackenzie *et al.*, 1997a).

Table 5.4. Oestrus symptoms scoring scale[1].

Oestrus symptoms	Points
Vulvular mucous discharge	3
Cajoling (flehmen)	3
Restlessness	5
Sniffing the vagina of another cow	10
Chin resting	15
Mounted but not standing	10
Mounting (or attempt) other cows	35
Mounting head side of another cow	45
Standing heat	100

[1] The assigned number of points was recorded each time a symptom was observed. If during two consecutive observation periods, the sum of the points exceeded 50, the heifer was considered to be in oestrus (Van Erdenburg *et al.*, 2002).

5.2.3 Chemical analysis

Fortnightly TMR, concentrate, and grass samples were bulked on a 6-monthly basis and mineralised wheat samples were bulked on a bi-monthly basis. Bulked feed samples were analysed according to AOAC (2012) for DM (934.01; intra-assay CV of 1.8%), crude protein (CP; 990.03; Section 3.1.1; intra-assay CV of 2.0%), ether extract (EE; 2003.05; intra-assay CV of 4.7%), and ash (942.05; Section 3.1.2; intra-assay CV of 1.1%). Concentrate and TMR samples were also analysed for starch (Section 3.1.6; intra-assay of 3.7% at Sciantic Analytical (Stockbridge Technology Centre, North Yorkshire, UK) according to ISO 6493 (2000). The neutral detergent fibre (NDF; Section 3.1.4) and acid detergent fibre (ADF; Section 3.1.4) content of the feeds was determined according to Van Soest *et al.* (1991); heat stable α-amylase was used for NDF determination (Sigma-Aldrich, Dorset, UK; intra-assay CV of 2.5% for NDF and 4.8% for ADF respectively). The water-soluble carbohydrate (WSC; Section 3.1.7) of the feeds was determined according to Thomas (1977; intra-assay CV of 5.5%). Fortnightly feed samples were analysed for dietary minerals by inductively coupled plasma-mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield,

UK; Section 3.1.8) following extraction using a DigiPREP digestion system (QMX Laboratories, Essex, UK). Total DM intake (kg DM/d) when housed was predicted according to Hoffman *et al.* (2008) using the equation:

$$\text{DMI (kg/d)} = 15.79 \times \left[1 - e^{(-0.00210 \times \text{BW})}\right] - 0.0820 \times \text{NDFdv} \qquad \text{Equation 5.1}$$

Where, $\text{NDFv} = (\text{dietary NDF as a \% of DM}) - \{22.07 + [0.08714 \times \text{BW}] - [0.00007383 \times (\text{BW})^2]\}$, and BW = live weight (kg). Dry matter intake at grass was predicted according to Minson and McDonald (1987) using the equation:

$$\text{DMI (kg/d)} = (1.185 + 0.00454\text{BW} - 0.0000026\text{L}^2 + 0.315\text{G})^2 \qquad \text{Equation 5.2}$$

Where, BW = live weight (kg), and G = growth rate (kg/d). Predicted total dry matter intakes calculated using equations are shown in figure 5.1. The recorded quantities of pelleted concentrate/mineralised wheat were then subtracted from the total estimated DM intakes to provide an approximate TMR or grass DM intake. Mean feed type proximate and mineral analyses are presented in Table 5.3, and were weighted to reflect the quantity of each feed type fed throughout the growing phase.

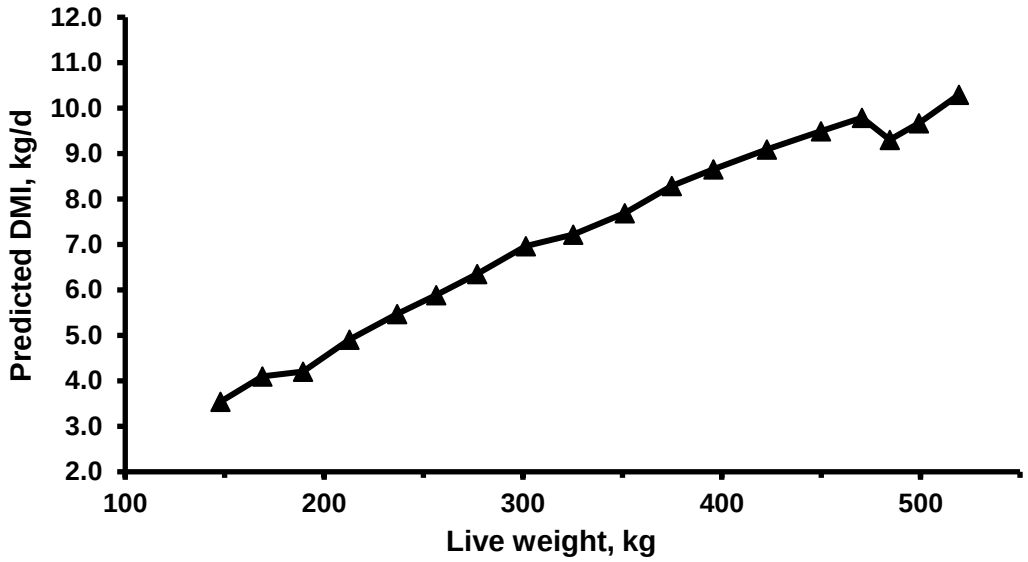

Figure 5.1. Predicted total dry matter intake (▲) of replacement dairy heifers between 4 and 22 months of age.

Plasma samples were analysed for AST and GGT (Randox Laboratories, Antrim, UK; kit catalogue no. AS1202, GT553; intra-assay CV of 0.4% and 1.9% respectively). Serum samples were analysed for Cp according to Henry *et al.* (1974; Section 3.2.3; intra-assay CV of 1.7%), and GLDH (Randox Laboratories, Antrim, UK; kit catalogue no. GL441; Section 3.2.5; intra-assay CV of 2.9%). Whole blood samples were analysed for SOD activity (Randox Laboratories, Antrim, UK; Kit catalogue no. SD 125; Section 3.2.4; intra-assay CV of 4.7%). The analysis of all plasma, serum and whole blood samples was conducted using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Haematological parameters were analysed using a Vet Animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK; Section 3.2.2). Plasma and liver samples were analysed for Cu, Fe, Zn and Mo by ICP-MS (Nexion 2000; Perkin Elmer, Beaconsfield, UK; Sections 3.2.6 and 3.3; intra-assay CV of 0.4%, 0.5%, 0.5%, and 1.3% for plasma, and 4.3%, 3.2%, 4.4%, and 3.8% for liver respectively) following a 1:50 dilution in 0.50 % concentrated nitric acid (Fischer Scientific, Loughborough, UK), 2.00 % HPLC grade methanol (Sigma-Aldrich, Dorset, UK) and 0.05% triton X-100 (Fischer Scientific, Loughborough, UK) adapted from Cope *et al.* (2009). Serum from the immune challenge was analysed for anti-ovalbumin antibody subclasses IgG_1 and IgG_2 (intra-assay CV of 7.9% for IgG_1 and 5.6% for IgG_2 respectively) by direct ELISA using mouse anti-bovine Immunoglobulin G_1 (IgG_1) and IgG_2 monoclonal antibodies (Bio-rad Laboratories, Hertfordshire), and goat anti-mouse IgG_2 conjugated to alkaline phosphatase (Bio-rad Laboratories, Hertfordshire).

5.2.4 Statistical analysis

Four heifers were removed from the experiment (two on the low treatment where one heifer died due to pneumonia and one contracted Q fever, and two on the high treatment where one heifer had a twisted spine and one was a tb reactor), and their data was excluded from the point of diagnosis. Continuous performance and blood parameters were analysed using repeated measures analysis of variance as a replicated block design. The model included the main effects of dietary Cu concentration and time, and their interaction according to:

$$Y_{ijkl} = \mu + B_i + C_j + T_k + F.T_{jk} + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of copper concentration (j = control or high); T_k = effect of time; $F.T_{jk}$ = interaction between copper concentration and time, and ε_{ijkl} = residual error.

Binomial data (e.g. conception rate) was analysed using linear model regression analysis under the logit function. The model included treatment as a term and significance was conveyed using chi-squared. In addition, the number of each semen type (bull) used at first service also included bull as a term, and its interaction with treatment (treatment + bull). Live weight and age at second conception were analysed by *t* test. Non-continuous performance parameters and liver mineral concentrations were analysed by analysis of variance as a replicated block design according to:

$$Y_{ijkl} = \mu + B_i + C_j + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of Cu concentration (j = control or high), and ε_{ijkl} = residual error.

All statistical analysis was conducted using Genstat version 18 (VSN International Ltd, Oxford, UK), and presented as the mean with the associated standard error of the differences of means or 95 % confidence interval; $P < 0.05$ was used as the threshold for significance and a trend was considered when $P < 0.1$. Week 0 values were used as a co-variate where appropriate.

5.3 Results

5.3.1 Dietary analysis, animal performance, and fertility

The mean DM content of the basal ration was 491 g/kg, with a crude protein, ash, and NDF content of 163, 88, and 497 g/kg DM respectively. The macro mineral content of the basal ration was 9.19, 4.61, 2.41, and 2.29 g/kg DM for Ca, P, Mg, and S respectively, and a micro mineral content of 16.2, 1.56, 85.3, and 285 mg/kg DM for Cu, Mo, Zn, and Fe respectively. The TMR, perennial ryegrass, and pelleted concentrate had mean Cu concentrations of 14.6, 7.8, and 35.9 mg/ kg DM, mean S concentrations of 2.22, 2.42, and 2.76 g/kg DM, and mean Mo concentrations of 1.6, 3.3, and 0.9 mg/kg DM respectively. The CuO boluses were estimated to supply an additional 15.6 mg Cu/kg DM to heifers on treatment H throughout the duration of the study.

The mean live weight and wither height of heifers on either treatment increased ($P < 0.001$) by 395 kg and 38 cm over the 18 months of the rearing phase, but there was no difference ($P > 0.05$) in either parameter at the beginning or end of the study (Table 5.5). There was however an effect of treatment ($P < 0.05$) on growth rate, with 0.79 kg/d for heifers on H, compared to 0.76 kg/d for those on C. Heifers on treatment H had both a higher mean condition score (CS; $P < 0.05$; Figure 5.3) of 2.93 and gain in CS ($P < 0.05$) of 0.26, compared to 2.86 and 0.09 for those on C over the duration of the study. There was also an effect ($P < 0.05$) of treatment on the age and live weight at which first observed oestrus occurred, with heifers on H reaching puberty 60 days earlier and 30 kg lighter than those on C (Table 5.6). In contrast, there was no effect ($P > 0.05$) of treatment on live weight at 1st or 2nd insemination, or indeed mean live weight when AI'd. Heifers on treatment H had a lower ($P < 0.01$) conception rate to 1st and 2nd service of 75% compared to 97% for those on C, which was reflected in an increased ($P < 0.05$) number of serves per conception of 1.9 for heifers on H compared to 1.4 for those on C. There was also no interaction ($P > 0.05$) between treatment and the number of each semen (bull) type used at first AI between the two treatments.

Table 5.5. Live weight, condition score, and wither height of replacement dairy heifers fed either a control or a high dietary copper concentration from 4 to 22 months of age.

	Cu supplementation level			
	C	H	SED	P-value
Initial live weight, kg	137	138	1.9	0.593
Final live weight, kg	527	537	7.0	0.154
Live weight Δ^1, kg/d	0.76	0.79	0.014	0.033
Initial condition score	2.93	2.89	0.037	0.405
Final condition score	3.01	3.17	0.063	0.018
Condition score Δ^1	0.09	0.26	0.072	0.025
Initial wither height, cm	100	100	0.8	0.657
Final wither height, cm	138	137	1.0	0.652
Wither height Δ^2, cm/d	0.08	0.08	0.002	0.489

[1] 4-22 months of age.

[2] 0-19 months of age.

Table 5.6. Fertility measures of replacement dairy heifers fed either a control or a high dietary copper concentration from 4 to 22 months of age.

	Cu supplementation level				
Item	C	H	SED	CI (95%)	P-value
1st observed oestrus[1], d	359	299	25.0		0.022
Live weight at 1st oestrus, kg	304	274	12.1		0.022
Pregnancy to 1st service[2] (%)	59.4	46.9		0.44,1.77	0.486
Pregnancy to 1st and 2nd service (%)	96.9	75.0		1.35, 6.68	0.008
Serves per conception	1.4	1.9	0.23		0.040
Age at 1st AI, d	468	468	15.3		0.959
Live weight at 1st AI, kg	411	410	10.6		0.926
Age at 2nd AI, d	511	512	18.8		0.959
Live weight at 2nd AI, kg	442	451	17.9		0.561
1st AI to conception, d	28	40	9.8		0.237
Mean AI live weight, kg	423	423	10.1		0.927
Age at calving, d	767	777	12.7		0.450
Calf birthweight[3], kg	38.8	40.4	1.1		0.170

[1] Birth to puberty onset.

[2] Semen type (Bull). Copper, P =0.901, bull, P <0.001, copper x bull = 0.200.

[3] Calf sex and breed considered as co-variates, breed had no effect and was subsequently removed.

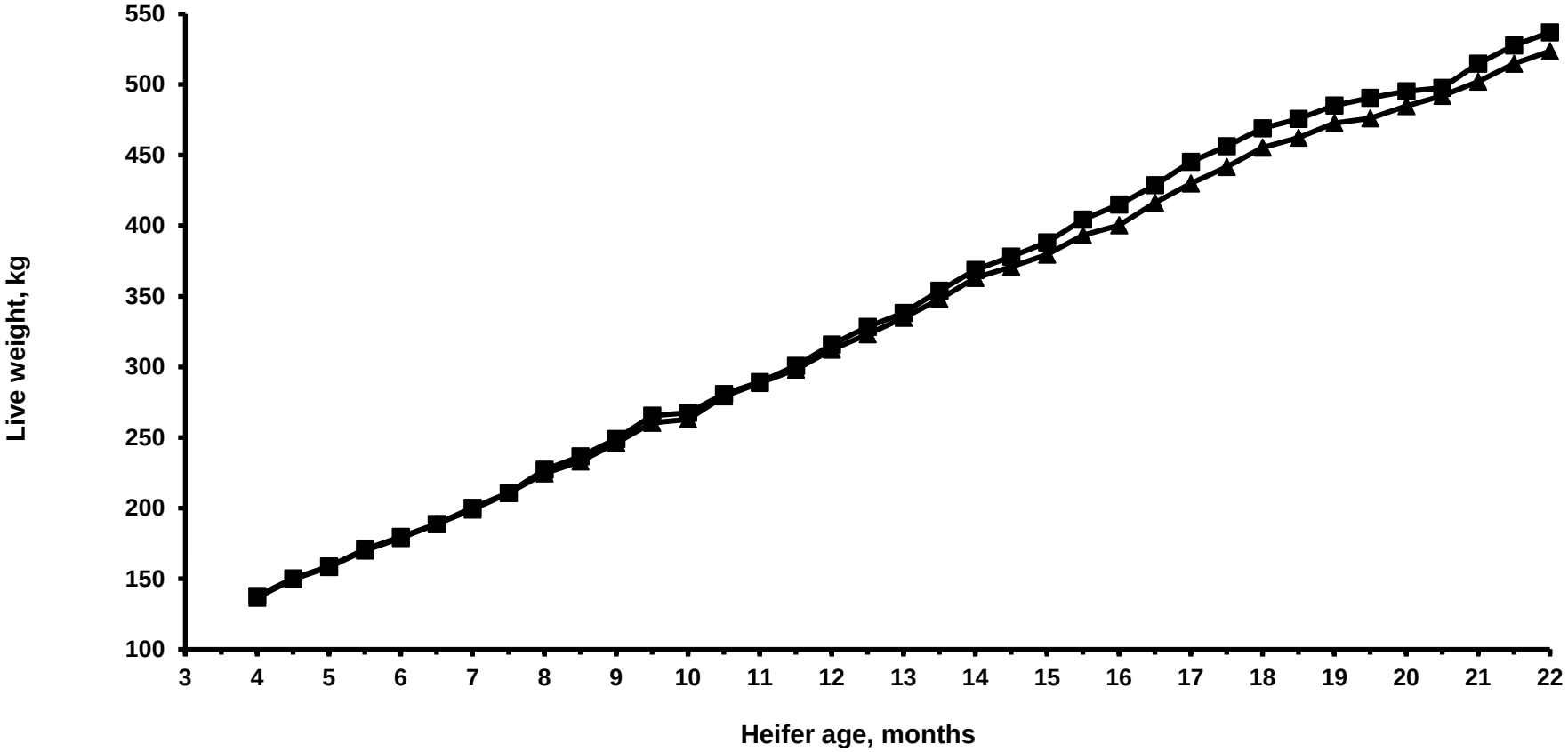

Figure 5.2. Fortnightly live weight of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. Pooled SED = 6.6. Copper, P = 0.160; time, P <0.001; copper x time, P = 0.274.

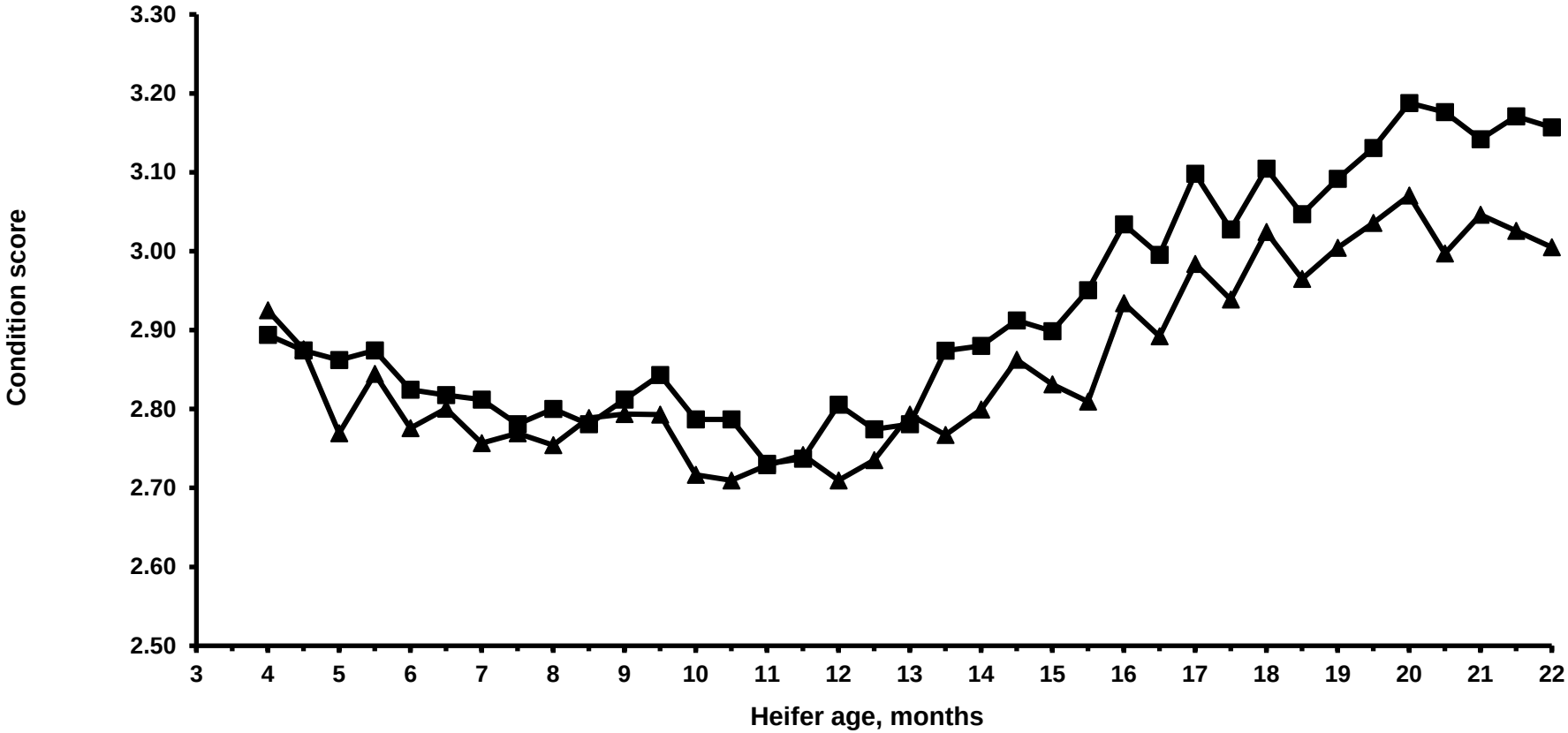

Figure 5.3. Fortnightly condition score of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. Pooled SED = 0.056. Copper, P = 0.027; time, P <0.001; copper x time, P = 0.442. Wk 0 used as a co-variate.

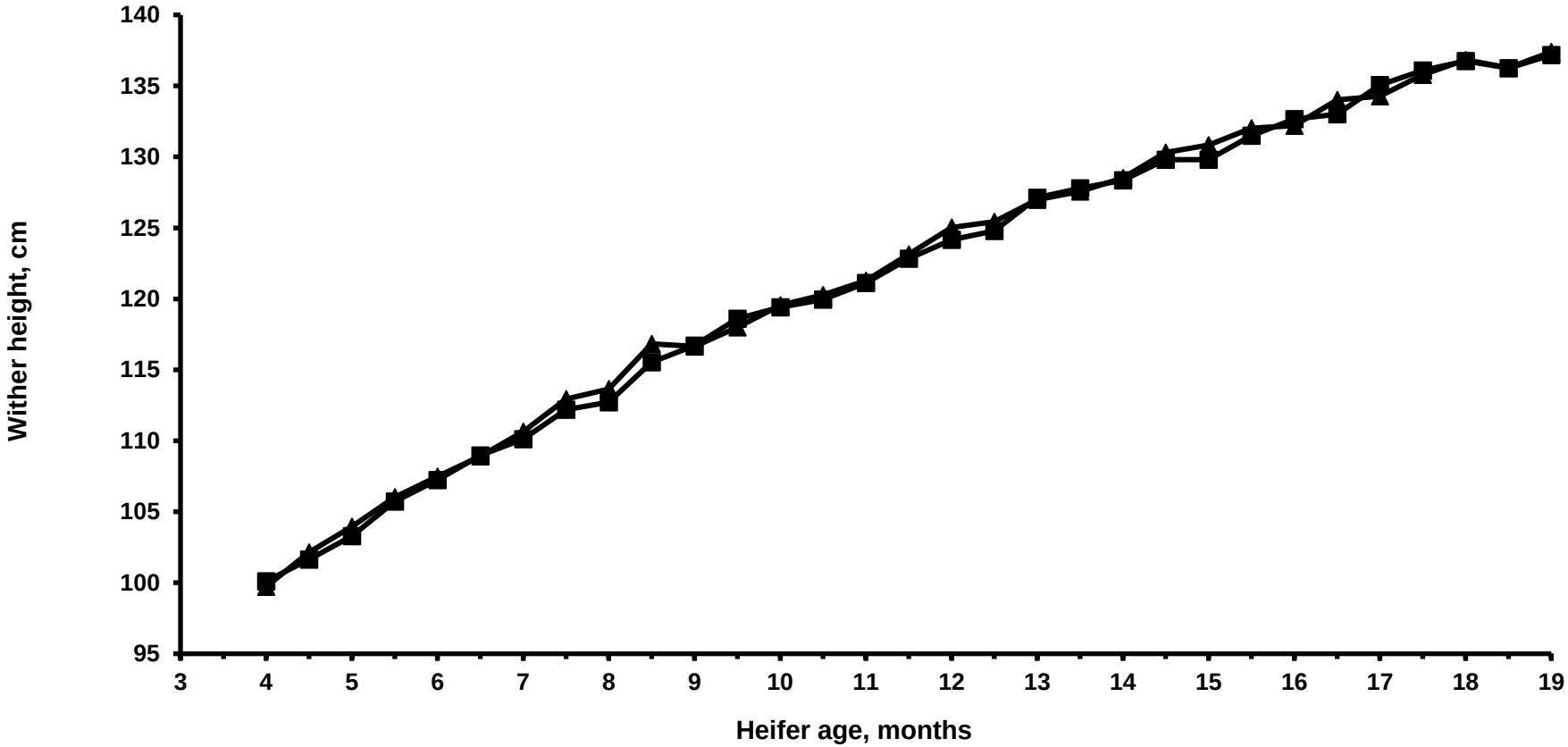

Figure 5.4. Fortnightly wither height of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. Pooled SED = 0.9. Copper, P = 0.650; time, P <0.001; copper x time, P = 0.844.

Table 5.7. Plasma mineral concentrations, copper-dependent enzymes, and liver function enzymes in replacement dairy heifers fed either a control or a high dietary copper concentration from 4 to 22 months of age.

	Cu supplementation level			P-values[1]		
Item[2]	C	H	SED	Cu	T	Cu x T
Plasma Cu, µmol/L	15.6	15.3	0.19	0.812	<0.001	0.966
Plasma Fe, µmol/L	33.4	34.5	0.92	0.231	<0.001	0.962
Plasma Zn, µmol/L	15.6	15.4	0.23	0.448	<0.001	0.508
Plasma Mo, µmol/L	0.25	0.26	0.041	0.862	<0.001	0.772
Plasma AST[4], U/L	74.1	83.7	3.72	0.018	<0.001	0.005
Plasma GGT[4], U/L	22.8	27.0	1.50	0.066	<0.001	0.091
Serum Cp, mg/dL	17.9	16.9	1.11	0.353	<0.001	0.801
Cp: Cu	1.16	1.09	0.073	0.955	<0.001	0.749
Serum GLDH[4,5], U/L	39.0	69.5	7.29	0.008	<0.001	0.014
SOD U/g of Hb	3658	3683	99.5	0.803	<0.001	0.438

[1] Cu= main effect of Cu source supplementation level, T = effect of time, Cu x T = interaction between supplementation level and time.
[2] 80 heifers used for plasma minerals (40 per treatment); 46 used for Cu-dependent and liver function enzymes (23 per treatment)
[3] Blood samples analysed as follows: plasma minerals = months 4 to 22, AST = months 4 to 10, GGT = months 4 to 10 and 22, GLDH = months 4 to 12 and 22, Cu-dependent enzymes (SOD, Cp, and Cp: Cu) = months 4 to 12, and 22.
[4] Normal range for liver function enzymes (Bidewell *et al.*, 2012); AST = 15-130 U/L, GGT = 0-30 U/L, and GLDH = 0-25 U/L.
[5] Week 0 used as a covariate.

5.3.2 Plasma mineral profile, Cu-dependent enzymes, and liver function enzymes

Plasma mineral concentrations were unaffected (P > 0.05) by dietary treatment (Table 5.7), with mean concentrations of 15.5, 34.0, 15.5, and 0.26 µmol/L for Cu, Fe, Zn, and Mo respectively. Plasma Cu (Figure 5.5a), Fe, and Zn concentrations were affected (P < 0.001) by time with all three fluctuating throughout the duration of the study. There was also an effect (P < 0.001) of time on plasma Mo concentration which remained relatively stable until 6 months of age (Figure 5.5b), before increasing to a peak concentration of 0.78 µmol/L for heifers on either treatment at 10 months, with a subsequent decline until 14 months of age, and remained relatively constant thereafter. Ceruloplasmin and SOD activity, as well as Cp: plasma Cu ratios were also unaffected (P < 0.05; Table 5.7) by treatment. There was however an effect (P < 0.001) of time on serum Cp activity (Figure 5.6a), which increased between 4 and 14 months of age with an intermittent spike at 6 months of age, and a subsequent decline after 14 months of age. Whole blood SOD activity increased (P < 0.001; Figure 5.6b) for heifers on either treatment from 4 months to a maximum at 8 months of age, with a sequential decrease at each successive measurement after 8 months of age. Heifers receiving treatment H had mean serum GLDH and plasma AST concentrations that were 30.5 U/L and 9.6 U/L higher (P < 0.05) than those on treatment C (Table 5.7). There

was also a trend (P < 0.1) for plasma GGT concentrations to be 4.2 U/L higher for heifers on treatment H compared to those on C. Time had an effect (P < 0.001) on AST (Figure 5.7), GGT and GLDH concentrations (Figure 5.8a,b), whereby all three were observed to decrease over the duration of the study. There was also a treatment x time interaction (P < 0.05) for serum GLDH and plasma AST, with concentrations of both enzymes decreasing for heifers on treatment C at the beginning of the study (4 to 8 months of age), whereas for those on H, concentrations increased at the beginning of the study (4 to 6 months of age) with a subsequent decrease thereafter.

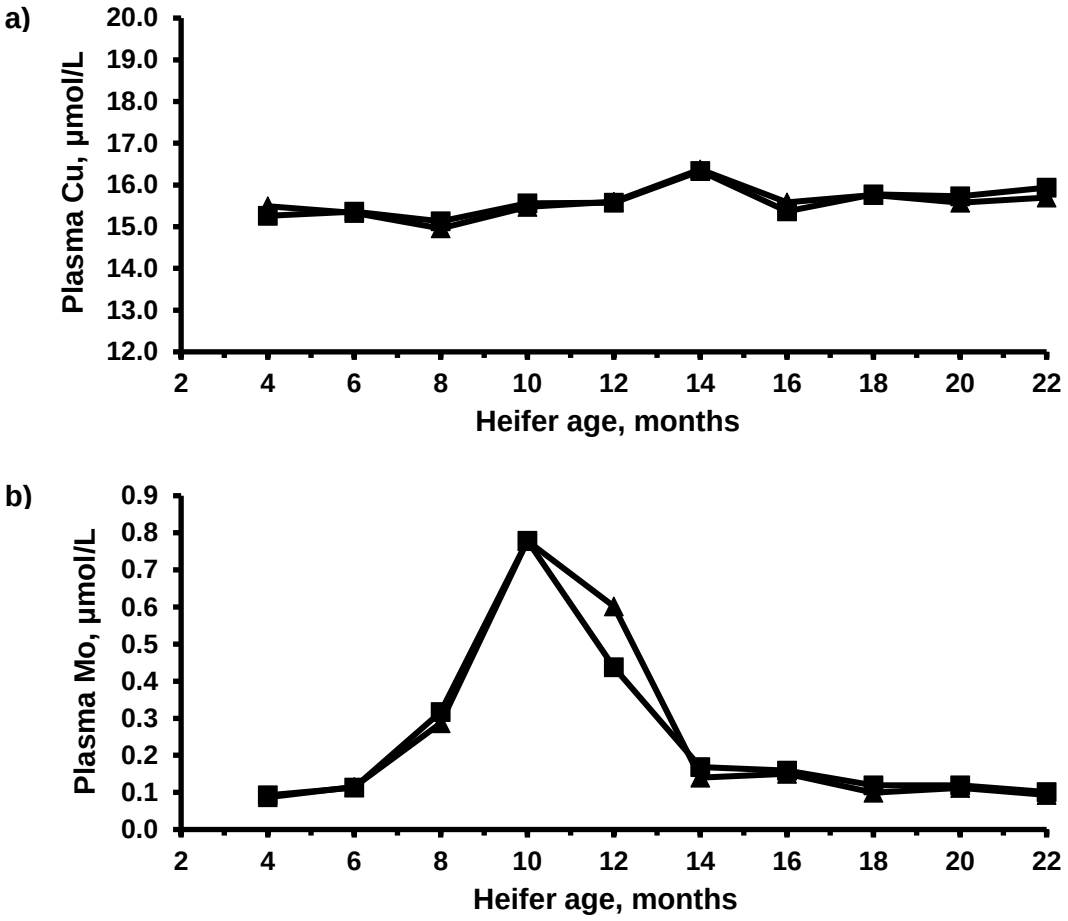

Figure 5.5. Plasma copper (a) and molybdenum (b) of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. For plasma Cu; pooled SED = 0.36. Copper, P = 0.812; time, P < 0.001; copper x time, P = 0.966. For plasma Mo; pooled SED = 0.101. Copper, P = 0.862; time, P < 0.001; copper x time, P = 0.772.

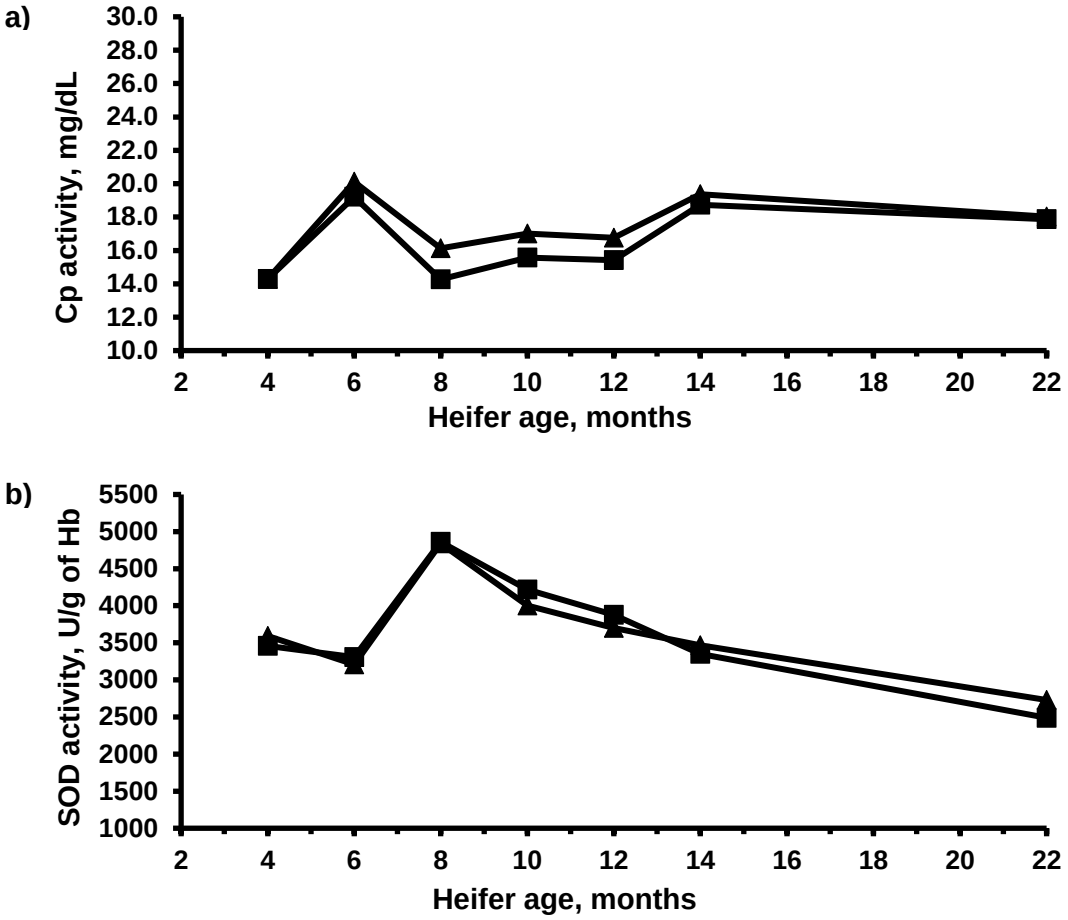

Figure 5.6. Serum ceruloplasmin activity (Cp; a) and whole blood superoxide dismutase activity (SOD; b) of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. For Cp; pooled SED = 1.55. Copper, P = 0.353; time, P <0.001; copper x time, P = 0.801. For SOD; pooled SED = 194.6. Copper, P = 0.803; time, P <0.001; copper x time, P = 0.438.

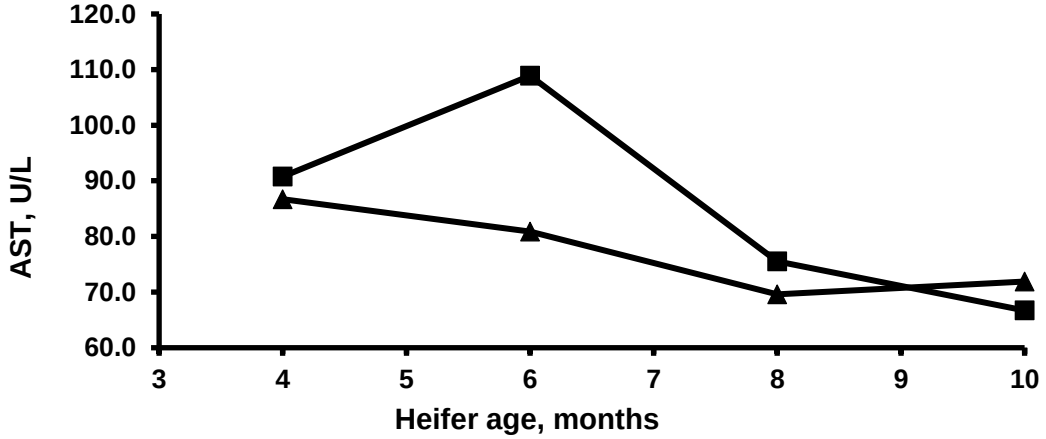

Figure 5.7. Plasma aspartate aminotransferase (AST) of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. Pooled SED = 6.29. Copper, P = 0.018; time, P <0.001; copper x time, P = 0.005.

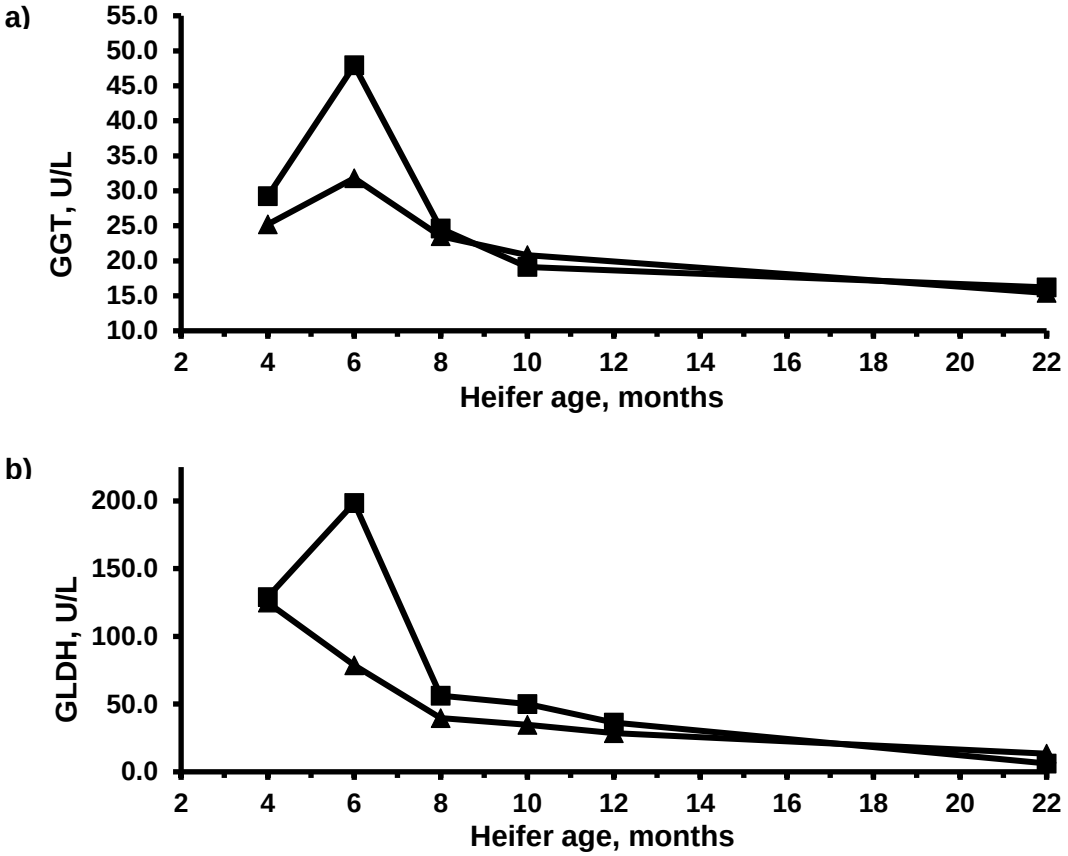

Figure 5.8. Plasma Ɣ-glutamyltransferase (GGT; a) and serum glutamate dehydrogenase (GLDH; b) of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration from 4 to 22 months of age. For GGT; pooled SED = 4.68. Copper, P = 0.066; time, P <0.001; copper x time, P = 0.091. For GLDH; pooled SED = 21.78. Copper, P = 0.008; time, P <0.001; copper x time, P = 0.014.

Table 5.8. Haematological profile of replacement dairy heifers fed either a control or a high dietary copper concentration from 4 to 22 months of age

	Cu supplementation level			P-values[1]		
Item[2]	C	H	SED	Cu	T	Cu x T
WBC, $10^3/mm^3$	12.2	11.5	0.35	0.054	<0.001	0.305
Mon No[3], $10^3/mm^3$	0.73	0.67	0.038	0.162	<0.001	0.630
Neu No, $10^3/mm^3$	5.11	4.89	0.191	0.264	<0.001	0.680
Lym No, $10^3/mm^3$	6.20	5.72	0.262	0.076	<0.001	0.286
Eo No, $10^3/mm^3$	0.16	0.20	0.021	0.176	<0.001	0.523
RBC, $10^6/mm^3$	8.42	8.35	0.202	0.755	<0.001	0.944
HCT, %	35.0	34.8	0.44	0.802	<0.001	0.929
Hb, g/dL	11.3	11.2	0.44	0.882	0.007	0.731

[1] Cu= main effect of Cu source supplementation level, T = effect of time, Cu x T = interaction between supplementation level and time.
[2] White blood cells (WBC), monocyte numbers (Mon No), neutrophil numbers (Neu No), lymphocyte numbers (Lym No), eosinophil numbers (Eo No), basophil percentage (Ba), red blood cell count (RBC), haematocrit percentage (HCT), and haemoglobin (Hb).
[3] Wk 0 values as a co-variate.

5.3.3 Haematological profile and ovalbumin immune challenge

There was a trend ($P < 0.10$) for heifers on treatment C to have 0.7 $10^3/mm^3$ and 0.48 $10^3/mm^3$ lower WBC and lymphocyte counts respectively (Figure 5.9), but there was no effect ($P > 0.05$) of treatment on any other haematological parameter (Table 5.8). In contrast, there was an effect ($P < 0.01$) of time on all haematological parameters which fluctuated throughout the duration of the study. Heifers on treatment H had a decreased anti-ovalbumin IgG_2 response ($P < 0.05$), in conjunction with an increased IgG_1 response ($P < 0.05$) compared to those on treatment C. There was also an effect ($P < 0.05$) of time on both the IgG_2 and IgG_1 response, where the responses to both classes of antibodies increased post-immunisation (Figure 5.10). There was also a trend ($P < 0.1$) for a time x treatment interaction with regard to the IgG_2 response, where the response of heifers on treatment C sequentially increased between day 0 and 63 post immunisation, but for heifers on treatment H, the IgG_2 response increased between 0 and day 14 post immunisation, with a decrease to day 28, followed by a gradual increase thereafter.

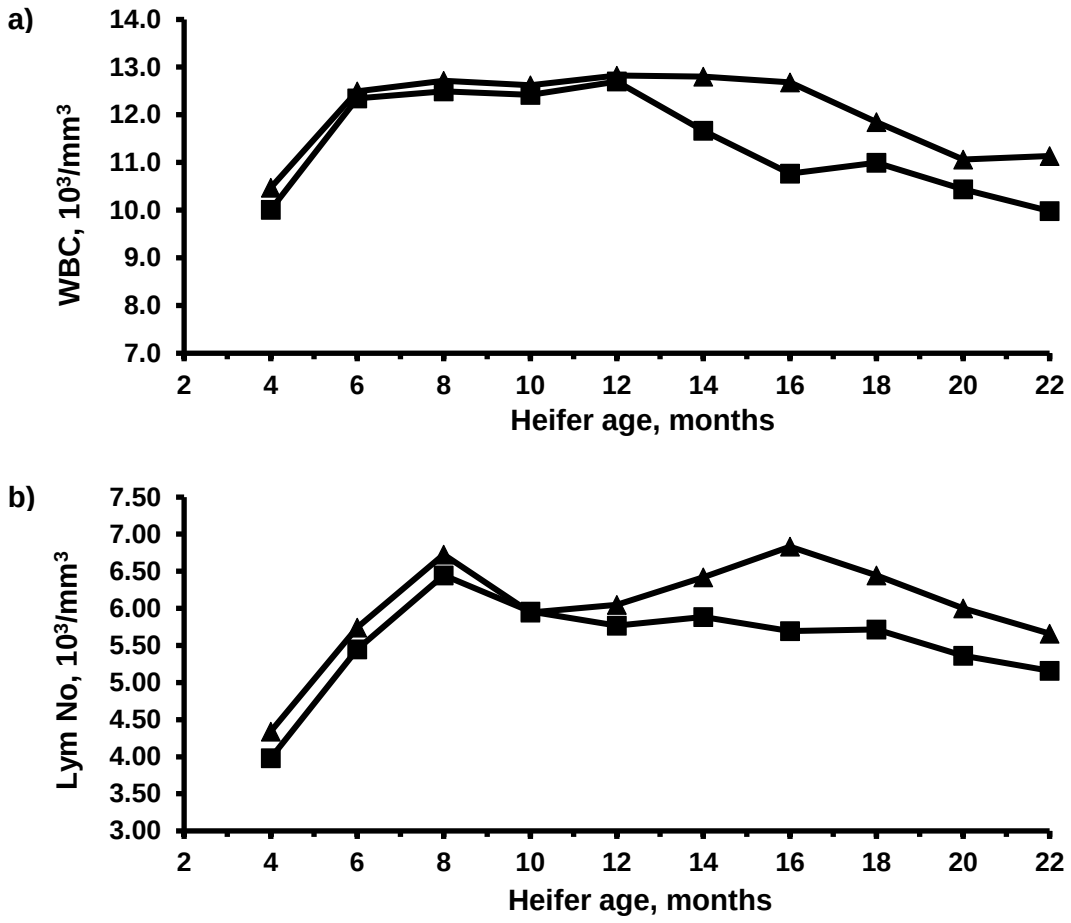

Figure 5.9. White blood cell (WBC; a) and lymphocyte number (Lym No; b) of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper concentration. For WBC; pooled SED = 0.63. Copper, P = 0.054; time, P <0.001; copper x time, P = 0.305. For Lym No; pooled SED = 0.381. Copper, P = 0.076; time, P <0.001; copper x time, P = 0.286.

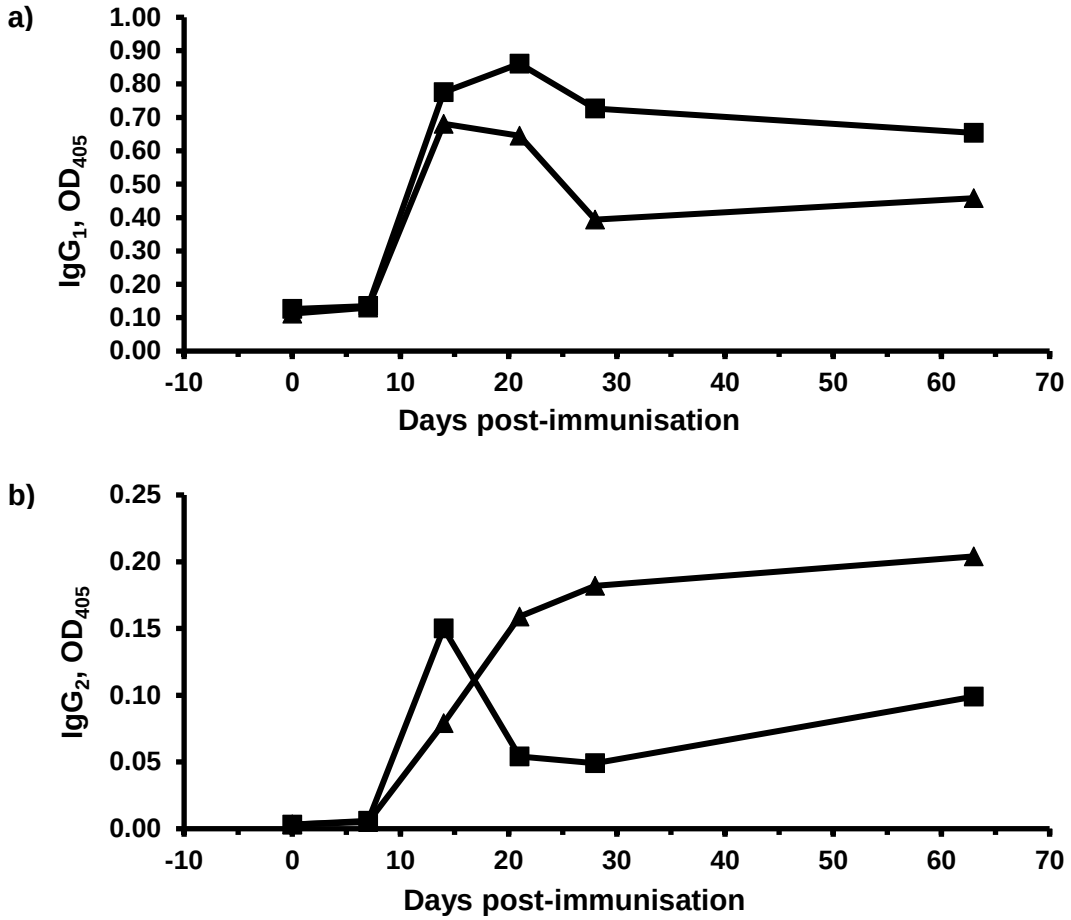

Figure 5.10. Anti-ovalbumin IgG_1 (a) and IgG_2 (b) antibody responses (mean OD_{405}) at 17 months old of replacement dairy heifers fed either a control (▲) or a high (■) dietary copper supplementation (12 heifers used; 6 per treatment). For IgG_1, pooled SED = 0.113. Copper, P = 0.033; time, P < 0.001; copper x time, P = 0.238. For IgG_2, pooled SED = 0.050. Copper, P = 0.031; time, P = 0.027; copper x time, P = 0.087.

5.3.4 Hepatic mineral concentrations

The highest hepatic Cu concentration was observed at 7 months of age for heifers on either treatment, with heifers on H having a higher (P < 0.01) concentration than those on C, with 798 and 643 mg/kg DM for H and C respectively (Table 5.9). There was subsequently a decrease (P > 0.05) in hepatic Cu for heifers receiving either treatment at 13 months of age to concentrations of 350 and 195 mg/kg DM for heifers receiving H and C respectively, which was still 155 mg/kg DM higher (P < 0.001) for those receiving H compared to C. This was followed by a further decrease (P > 0.05) in hepatic Cu concentration for heifers on either treatment to 293 and 128 mg/kg DM for those on H and C respectively six weeks prior to calving. Heifers on H continued to maintain a 165 mg/kg DM higher (P < 0.001) hepatic Cu concentration compared to those on C. Finally, hepatic Fe, Mo, and Zn concentrations and their change between sampling points were unaffected (P > 0.05) by dietary treatment throughout the duration of the study.

Table 5.9. Liver mineral concentrations of replacement dairy heifers fed either a control or a high dietary copper concentration from 4 to 22 months of age.

	Cu supplementation level			
Item[1]	C	H	SED	P-value
7 months Cu, mg/kg DM	643	798	47.7	0.002
13 months Cu, mg/kg DM	195	350	22.4	<0.001
Cu Δ (7-13 months), mg/kg DM/d	-2.71	-2.71	0.227	0.994
Pre-calving Cu[2], mg/kg DM/d	128	293	27.0	<0.001
Cu Δ^2 (13 months- pre-calving), mg/kg DM/d	-0.14	-0.10	0.067	0.555
7 months Fe, mg/kg DM	329	330	57.3	0.989
13 months Fe, mg/kg DM	371	310	34.1	0.087
Fe Δ (7-13 months) mg/kg DM/d	0.25	-0.11	0.393	0.366
Pre-calving Fe[2], mg/kg DM/d	394	421	39.2	0.513
Fe Δ^2 (13 months- pre-calving) mg/kg DM/d	0.16	0.45	0.235	0.261
7 months Mo, mg/kg DM	2.83	3.63	0.506	0.128
13 months Mo, mg/kg DM	3.30	3.67	0.223	0.105
Mo Δ (7-13 months), µg/kg DM/d	2.8	0.3	3.36	0.458
Pre-calving Mo[2], mg/kg DM/d	3.42	3.30	0.144	0.423
Mo Δ^2 (13 months- pre-calving), µg/kg DM/d	1.0	0.4	0.36	0.149
7 months Zn, mg/kg DM	94.9	84.4	13.61	0.446
13 months Zn, mg/kg DM	79.1	83.8	5.6	0.407
Zn Δ (7-13 months), mg/kg DM/d	-0.096	-0.004	0.073	0.218
Pre-calving Zn[2], mg/kg DM/d	127	115	12.1	0.367
Zn Δ^2 (13 months- pre-calving), mg/kg DM/d	0.16	0.12	0.029	0.243

[1] Pre-calving biopsies were taken 6 weeks prior to calving.

[2] Liver biopsies were taken from all 80 heifers (40 per treatment) at 13 and 7 months of age respectively, whereas only 12 (6 per treatment) were sampled prior to calving.

5.4 Discussion

5.4.1 Performance and fertility

This study is the first to examine the long-term effects of dietary Cu concentration on replacement Holstein Friesian heifer performance, health and fertility. To date, studies have focused primarily on beef cattle (Engle, 2014), been short-term in duration (Engle and Spears, 2000), and generally not monitored health and fertility (Arthington, 2005). The basal ration (C) had a mean Cu concentration of 16.2 mg/kg DM over the 18-month duration of this study, which was similar to the target of 15 mg/kg DM predicted to meet the heifer's requirements (ARC, 1980; NRC, 2001; Table 2.4). Assuming the manufacturers stated rate of release (Agrimin Ltd, Kirmington, UK), the CuO boluses were predicted to provide an additional 15 mg/kg DM to heifers on treatment H. Based on estimated DM intake (Minson and McDonald, 1987; Hoffman *et al.*, 2008), heifers on treatment H received an additional 15.6 mg/kg DM over the duration of the study. This resulted in a total dietary Cu concentration of 31.8 mg/kg DM, which was similar to the mean dietary concentration of 28 mg/kg DM fed in the UK over the winter to dairy cows on 50 survey farms (Sinclair and Atkins, 2015). Additional Cu was supplied to heifers on treatment H as CuO, whereas additional Cu tends to be added to diets as inorganic $CuSO_4$ due to its lower cost (Brown and Zeringnue, 1994), and increased apparent availability in comparison to inorganic CuO powder (Kegley and Spears, 1994). Kegley and Spears (1994) reported a relative bioavailability of 7 % for CuO powder compared to $CuSO_4$ powder when the latter was used as a reference source. The CuO supplied by the boluses to heifers on treatment H may therefore be not comparable to the $CuSO_4$ sources likely to be supplied on farms surveyed by Sinclair and Atkins (2015), and that the decrease in apparent availability of CuO means the dietary concentrations are not comparable (Kegley and Spears, 1994). Copper oxide was in this study was supplied as an intra-ruminal bolus containing CuO oxide needles (Agrimin Ltd, Kirmington, UK), rather than a powder. Yost *et al.* (2002) reported no effect of Cu source on final hepatic Cu concentration when growing Holstein-Friesian heifers received additional Cu either as 15 mg/kg DM of $CuSO_4$ powder or as an intra-ruminal bolus containing 20 g of CuO needles (Copasure, Animax Ltd), with the CuO bolus estimated to supply approximately 13.4 mg of Cu/kg DM across the duration of the study (Hoffmann *et al*., 2008). The difference in Cu availability when CuO is supplied as a needle compared to powder a form may be due to the increased passage rate of powder through the digestive tract of the animal (Langland *et al.*, (1989). In contrast, CuO needles remain embedded in the gastrointestinal tract for a longer period of time, allowing increased Cu solubilisation and absorption (Kegley and Spears, 1994). Discrepancies in absorption and metabolism due to the CuO source supplied in this study in comparison to $CuSO_4$ used predominantly on

commercial farms may therefore have been minimal (Brown and Zeringue, 1994; Yost *et al.*, 2002).

Dietary Cu absorption is not only affected by the form and level of supply but is also related to interactions with dietary antagonists such as S, Mo, and Fe (Suttle, 2010). The mean diet had a Mo concentration that was 0.6 mg/kg DM higher than the concentration of 1.1 mg/kg DM reported by Sinclair and Atkins (2015). The difference was mainly due to the elevated Mo concentration in both the grazing sward and TMR in this study (Atkins *et al.*, 2018; Sinclair and Atkins, 2015). In contrast, the S concentration of the mean diet (2.3 g/kg DM) was typical of that previously fed in other dairy cow studies at Harper Adams University (Sinclair *et al.*, 2013). The Fe concentration of the mean diet was 31 mg/kg DM lower than the mean of 316 mg/kg DM reported by Sinclair and Atkins (2015) in diets-fed to UK dairy cows in winter. Heifers in each pair in the current study were fed the same basal ration throughout and so would have been exposed to the same concentrations of dietary antagonists, and any observed treatment effects should therefore be a result of dietary Cu concentration.

Heifers were fed throughout the study to achieve a growth rate of 0.8 kg/d (AFRC, 1993). Growth rates below this threshold increase the costs associated with heifer rearing and the period of time taken for a return of investment (Bach, 2011; Boulton *et al.*, 2015). Growth rates in excess of this threshold have been shown to negatively affect prepubertal mammary gland development and subsequently reduce milk yield (Sejrsen *et al.*, 1983). Despite receiving the same basal ration throughout the study, heifers on treatment H had a higher growth rate of 0.79 kg/d, which was closer to the target of 0.8 kg/d compared to heifers on treatment C at 0.76 kg/d. Literature is conflicting regarding the effects of Cu supplementation on growth rate, Ward *et al.* (1993) reported no effect of Cu supplementation on the growth rate of crossbred steers during the growing phase, when a basal ration containing 6.2 mg Cu/kg DM was supplemented with 10 mg of Cu/kg DM as $CuSO_4$ to provide a total dietary concentration of 16.2 mg Cu/kg DM. In contrast, Arthington (2005) reported that the growth rate of crossbred heifers decreased by 0.04 kg/d, when a basal diet containing 7.8 mg Cu/kg DM was supplemented with 120 mg Cu/kg DM as $CuSO_4$ to provide a total concentration of 127.8 mg Cu/kg DM. Similar to the findings of this study however, Ward and Spears (1997) reported that the growth rate of Angus steers increased (by 0.17 kg/d), when a basal ration containing 5.2 mg Cu/kg DM was supplemented with 5.0 mg of Cu/kg DM as $CuSO_4$ to provide a total dietary concentration of 10.2 mg Cu/kg DM.

Reasons for the increased growth rate of heifers on treatment H in this study are unclear but may relate to increased feed intake and/or digestibility (Arthington and Spears, 2007; Durand and Kawashima, 1980). Arthington and Spears (2007) reported an increase in the DMI of crossbred heifers, when a basal diet containing 6.7 mg of Cu/kg DM was supplemented with 15 mg of Cu/kg DM as $CuSO_4$ to provide a total dietary concentration of 21.7 mg Cu/kg DM. Peptidyglycine α-amidating monooxygenase (PAM) is a Cu-dependent enzyme required for the generation of the appetite-regulating hormones gastrin and cholecystokinin (Prohaska, 2012; Suttle, 2010). Ruminant studies examining the effects of Cu status on PAM are scarce, but both the serum and tissue activity of PAM in rats has been positively correlated with dietary Cu intake (Prohaska *et al.*, 2005; Suttle, 2010). Dietary Cu concentration may also influence digestibility, Durand and Kawashima (1980) reviewed the effects of dietary Cu concentration on ruminal fermentation both *in vitro* and *in vivo*, and concluded that Cu supplementation in excess of the animal's requirements can have both positive and negative effects upon cellulolysis depending on the inclusion rates of other minerals and the type of basal ration fed. Lopez-Guisa and Satter (1992) also reported increased DM disappearance from the rumen of Holstein heifers when a basal ration (Cu inclusion not disclosed) was supplemented with 9.9 mg of Cu/kg DM as $CuSO_4$ and 0.25 mg of Co/kg DM as $CoSO_4$. One possible reason for this change in DM disappearance was an increased attachment of bacteria to plant material (Storry, 1961), where Cu and Co as divalent cations bridge the anionic charges between the bacterial and plant cell walls (Sorners, 1983). There is also the possibility that dietary inclusion of these cations in excess of the animal's requirements resulted in increased microbial growth (Young, 1979). Intake and digestibility however are not unrelated, Oba and Allen (1999) reported that DMI increased with NDF digestibility due to an increased disappearance of fiber from the rumen (Allen and Oba, 1996). It is however difficult to speculate whether the additional Cu received by heifers on treatment H altered intake and/or ruminal fermentation as neither variable was monitored in this study, however there are several mechanisms by which either or both may have been affected (Lopez-Guisa and Satter, 1992; Prohaska, 2012).

The greater increase in CS (0.17) over the study period for heifers on treatment H compared C is not surprising given that Holstein heifers have been shown to increase the ratio of fat deposition to lean tissue formation at increased growth rates (Van Amburgh *et al.*, 1997). Atkins *et al.* (2018) reported a CS increase of 0.08 over 3 months at a growth rate of 1.24 kg/d compared to a CS decrease of -0.06 at a growth rate of 0.95 kg/d when Holstein-

Friesian replacement heifers were out-wintered on fodder beet compared to grass silage. There may however be other factors contributing to the difference in condition score in the current study (Ward and Spears, 1997; Yang *et al.*, 2019). Ward and Spears (1997) identified the potential of Cu status to alter lipid metabolism and reported leaner carcasses with decreased backfat at the 12th rib when Angus steers were supplemented with Cu as $CuSO_4$ throughout the receiving (7.5 mg of Cu/kg DM; basal ration contained 6.89 mg of Cu/kg DM), growing (5.0 mg of Cu/kg DM; basal ration contained 5.2 mg of Cu/kg DM), and finishing (5.0 mg of Cu/kg DM; basal ration contained 2.85 mg of Cu/kg DM) phases respectively, despite an increased growth rate of steers receiving supplemental Cu in the finishing phase (Ward and Speers, 1997). Additionally, Engle (2014) extensively reviewed the effects of Cu supplementation on subcutaneous adipose tissue metabolism in beef cattle, and concluded that there was the potential for supplemental Cu to reduce subcutaneous adipose tissue depth with a ceiling on this effect at 20 mg of Cu/kg DM regardless of Cu source (Engle and Spears, 2000). It is therefore surprising that a higher CS was observed in heifers receiving a higher dietary Cu concentration in the current study, but it should be taken into consideration that the Cu concentrations of the basal rations (ranging from 2.85 to 10.2 mg of Cu/kg DM) in the studies reviewed by Engle (2014) were lower than the 16.2 mg Cu/kg DM fed to heifers in the current study (Engle and Spears, 2000; Ward and Spears, 1997; Engle *et al.*, 2000).

There is evidence in other species relating an increased Cu status and lipid deposition (Gu *et al.*, 2019; Yang *et al.*, 2019). In the human population, elevated plasma Cu and Cp have been linked to both increased lipid deposition and obesity, with several possible explanations relating to oxidative stress and inflammation (Gu *et al.*, 2019; Habib *et al.*, 2015). These explanations seem unlikely given the lack of an effect of Cu supplementation on Cp or plasma Cu in the current study. An explanation may however reside in the elevated hepatic Cu concentration of heifers on treatment H (Roche *et al.*, 2009; Roughead and Lukaski, 2003). Growth hormone (GH) is released from the anterior pituitary under negative feedback regulation by hepatically synthesised insulin-like growth factor 1 (IGF-1; Roche *et al.*, 2009). The resulting net effect of a decrease in GH is a partitioning of nutrients towards adipocytes (Liesman *et al.*, 1995; del Rincon *et al.*, 2007). Studies in ruminants are scare, but serum IGF-1 concentrations have been positively correlated with dietary Cu intake in rats (Roughead and Lukaski, 2003), whilst increasing Cu concentrations were shown to increase the expression of IGF-1 mRNA in the chondrocytes of newborn pigs *in vitro* (Wang *et al.*, 2012). Additionally, Ward and Spears (1997) suggested the potential of Cu status to affect circulating IGF-1 in beef heifers but did not declare the nature (increase vs decrease) of this effect. These observations lead to the hypothesis that the increased hepatic Cu

concentration in heifers on treatment H may have increased hepatic secretion of IGF-1 (Roughead and Lutaski, 2003), decreasing circulating GH concentrations, and subsequently decreasing subcutaneous adipose tissue deposition (Roche, 2007; Roche *et al.*, 2009).

The onset of puberty in the large dairy breeds normally occurs at approximately 9-11 months at live weights ranging from 250 to 280 kg (Sejrsen, 1994). Nutrition is thought to play a major role in the age at which puberty onset occurs. Foldager *et al.* (1988) varied heifer growth rate through plane of nutrition, and reported a decrease in age at first observed oestrus from 16.4 to 8.4 months as growth rate increased from 0.40 to 0.85 kg/d, however the mean live weight at which first observed oestrus occurred was unaffected by heifer growth rate (Foldager *et al.*, 1988). This confirmed that the onset of puberty in cattle is closely related to body development as opposed to chronological age (Schillo *et al.*, 1992). It is therefore surprising that heifers on treatment H not only reached puberty 60 days earlier but at a 30 kg lower live weight than those receiving C. There is however some evidence linking Cu status and oestrus onset (Ahmed *et al.*, 2009; Phillippo *et al.*, 1987b). Phillippo *et al.* (1987b) reported that the onset of puberty was delayed by 12 weeks when Hereford-Friesian heifers fed a basal diet containing 4, 100, and 0.1 mg/kg DM of Cu, Fe, and Mo respectively, was supplemented with 5 mg Mo/kg DM to provide a total dietary concentration of 5.1 mg Mo/kg DM. Phillippo *et al.* (1987b) concluded however that this was most likely an effect of feeding supplemental Mo as opposed to the associated decreased Cu status. Indeed, heifers in the study of Phillippo *et al.* (1987b) were fed a third diet with a total Fe inclusion of 600 mg/kg DM and experienced the same decline in Cu status as those fed the Mo supplemented diets, but oestrus onset and fertility were unaffected. In contrast, Ahmed *et al.* (2009) investigated malnutrition on small holder Egyptian Buffalo farms and reported that 21.8 % of hypocupric Egyptian Buffalo suffered from ovarian inactivity. Hypocuprosis of heifers on treatment H seems unlikely given that hepatic Cu concentrations of 195 mg/kg DM at 13 months of age were well in excess of the 19 mg/kg DM considered to pose a deficiency risk (Laven and Livesey, 2005), and were indeed higher than those receiving C. There may be a more plausible explanation for the earlier onset of puberty in heifers on treatment H relating to the hypothalamic-pituitary-ovarian axis (Schillo *et al.*, 1992). The pulsatile release of luteinising hormone required for normal ovarian cyclicity is inhibited by ovarian oestrogen (Day *et al.*, 1984). The transition of the animal into adulthood reduces this inhibition and results in increased follicular growth rate and normal ovarian cycling (Foster and Ryan, 1981). It has been identified that IGF-1 may serve to promote early follicular development (Gong, 2002), and as such its administration has been associated with the earlier onset of puberty (Adashi *et al.*, 1985). This may explain the decreased age

at first oestrus of heifers on treatment H, if higher hepatic Cu concentrations increased circulating IGF-1 concentrations (Roughead and Lukaski, 2003), however plasma IGF-1 was not monitored in the current study.

Evidence is conflicting regarding the effects of growth rate on heifer conception rate (Leaver, 1977; Bourne *et al.*, 2007). Leaver (1977) reported no difference in conception rate when growth rates increased from 0.34 to 0.68 kg/d in British Friesian heifers. In contrast, Bourne *et al.* (2007) investigated the effects of heifer size and growth rate on age at first calving and concluded that dairy heifers who failed to conceive at 15 months of age were 26 kg lighter at 9 months of age than those that did conceive. Despite the decreased conception rate to 1st and 2nd insemination (96.9% vs 75% for treatments C and H), leading to a decreased number of serves per conception (1.4 vs 1.9 for treatments C and H) for heifers on treatment C in the current study, there was no difference in heifer live weight at 1st or 2nd insemination, or indeed mean live weight at AI, indicating that Cu does not affect conception by affecting growth rate. This is not the first study to identify decreased conception rates as a result of Cu supplementation, Hawkins (2014) reported a decrease in 21-day pregnancy rates (43% vs 47% for control and treatment cows) when New Zealand Friesian x Jersey cows on seven farms received 200 mg of Cu as a Ca Cu EDTA injection 10 days before their mating start date, although basal dietary Cu concentration and/or indicators of Cu status were not monitored (Hawkins, 2014). Hawkins (2014) did not speculate on the biological mechanisms by which this depression in conception rate may have occurred, but there is some evidence of both direct and indirect effects of dietary Cu concentration on conception (Matsubayashi *et al.*, 2017; Starbuck *et al.*, 2006). Matsubayashi *et al.* (2017) reported that elevated serum Cu concentrations were a risk factor for implantation failure within the human population of the Japanese Osaka region, where elevated serum Cu concentrations were thought to be due to dietary Cu intakes (0.97 mg/d; MHLW, 2015a) in excess of requirements (0.90 mg/d; MHLW, 2015b). One reason suggested for this decline in fertility was a deposition of copper ions within the endometrium of the uterus resulting in implantation failure (Oster and Salgo, 1975). There may however be another possible explanation for decreased conception rates relating to the potential effects of hepatic Cu on the hypothalamic-pituitary-ovarian axis discussed previously (Schillo *et al.*, 1992). Treatment of Holstein and Ayrshire dairy cows with a single dose of growth hormone (rbST) 12 hours post-insemination was shown to increase conception rates from 25.8% to 64.3% for animals in excess of 100 days in milk (Starbuck *et al.*, 2006). It is thought that GH increases progesterone concentrations in dairy cattle post insemination with a subsequent increase in successful implantation and pregnancy retention (Morales-Roura *et al.*, 2001; Starbuck *et al.*, 2004). If indeed, circulating IGF-1 concentrations were

increased due to higher hepatic Cu concentrations in heifers on treatment H (Roughead and Lukaski, 2003), the reduction in GH may have resulted in decreased progesterone concentrations and lower conception rates in these animals (Morales-Roura *et al.*, 2001; Roche *et al.*, 2009). Neither circulating IGF-1 concentrations, plasma progesterone, nor the Cu content of endometrium were monitored in the current study, and so conclusive biological mechanisms for these effects upon fertility cannot be made.

5.4.2 Plasma mineral profile, Cu mediated enzymes, and the immune response

Similar to other studies that have examined the effects of dietary Cu concentration on Cu status (Arthington *et al.*, 2005; Yost *et al.*, 2002), plasma Cu concentration was unaffected with a mean value of 15.5 µmol/L for heifers on both C and H, which was well in excess of the lower limit of 9 µmol/L considered to be adequate (Laven and Livesey, 2005). This finding supports previous observations that it is only in cases were the animal is experiencing excessively high or low hepatic Cu concentrations that plasma Cu concentration becomes a useful indicator of Cu status (Dias *et al.*, 2013; Sinclair *et al.*, 2017). Plasma Mo concentration was also unaffected by dietary Cu concentration but was observed to increase between 8 and 14 months of age (Figure 5.5), reaching a maximum of 0.78 µmol/L for heifers on either treatment at 10 months of age. Given that dietary Mo concentrations were 2.0 times higher in the perennial ryegrass compared to the TMR in the current study (Table 5.3), it is not surprising that plasma Mo concentrations increased during the heifers first grazing season (8 to 14 months of age) as dietary Mo is readily absorbed into the bloodstream (Suttle, 1991; Sinclair *et al.*, 2017). Due to the unresponsiveness of plasma Cu to dietary Cu, other indicators of Cu status including the activity of the Cu-containing enzymes Cp and SOD have been investigated (Suttle and McMurray, 1983; Humphries *et al.*, 1983), along with the ratio of Cp to plasma Cu (Cp: Cu; Mackenzie *et al.*, 1997b). In the current study, there was no effect of treatment on any of these indicators of Cu status. It is surprising that Cp activity was lower in heifers on either treatment at the beginning of the study (4-12 months of age), when liver Cu concentrations were observed to be at their highest. There are two potential reason for this decreased Cp activity, one being decreased Cp synthesis due to hepatobiliary disease at high liver Cu concentrations (Gault *et al.*, 1966), or decreased Cp synthesis in juveniles relative to their adult counterparts (Chang *et al.*, 1975), although evidence for this effect in juvenile ruminants is scarce (Suttle, 2010). It may however be more plausible given that the effects of hepatobiliary disease upon Cp synthesis are inconsistent (Chang *et al.*, 1975).

The production of the immunoglobulin isotopes IgG_1 and IgG_2 is widely used as an indicator of the underlying Thymus helper cell (Th-cell) balance within mammals during immunostimulation (Firacative *et al.*, 2018), where a Th1 response induces the production of IgG_2, and a Th2 response induces the production of IgG_1 (Firacative *et al.*, 2018). The current study is not the first to report an increased Th2 response as a result of increasing dietary Cu concentration, Ward and Spears (1999) also observed an increase in Th2 response to ovalbumin immunisation in growing Angus steers. When a basal diet containing 5.2 mg Cu/kg DM was supplemented with 5 mg Cu/kg DM as $CuSO_4$ to provide a total dietary Cu concentration of 10.2 mg Cu/kg DM for the Cu supplemented diet (Ward and Speers, 1999). Although Ward and Spears (1999) did not monitor the Th1 response to ovalbumin immunisation, it was observed to decrease for heifers on treatment H in the current study. This may have consequences for the cattle industry as it has been demonstrated that the development and maintenance of a sustained Th1 response is associated with decreased pathology and greater control of *Mycobacterium bovis* (Welsh *et al.*, 2005). Ward and Spears (1999) did not speculate on the relationship between dietary Cu supply and the immune system, but an explanation may reside with the increased hepatic Cu concentration in heifers receiving the high dietary Cu concentration (McElwee *et al.*, 2009). Nuclear factor kappa light chain enhancer of B cells (NF-κB) is a transcriptional factor that upon activation mediates the prevention or induction of allergic, degenerative, or chronic inflammatory diseases (Kudrin, 2000). The influence that Cu exerts on the action of NF-κB seems to be cell type specific (McElwee *et al.*, 2009), in most of the cells of the immune system Cu is thought to have an inhibitory effect (McElwee *et al.*, 2009), whereas in hepatic cells Cu is thought to activate NF-κB (Cisternas *et al.*, 2005). The activation of NF-κB within the liver has been associated with both increased hepatic and circulating concentrations of transforming growth factor β (TGF-β; Lu *et al.*, 2016; Nagarajan *et al.*, 2000). Transforming growth factor-β serves to prevent the development of autoimmune disease without compromising the immune response (Wrzesinski *et al.*, 2007), it's subsequent potential influence on the immune system includes the inhibition of T-cell proliferation (Kehrl *et al.*, 1986), and the attenuation of the production of interferon-γ (IFN-γ), an essential cytokine for the stimulation of the Th1 response (Bellone *et al.*, 1995; Rook *et al.*, 1986). The increased hepatic Cu concentrations of heifers on the high dietary Cu concentration may therefore have increased circulating TGF-β which subsequently decreased both T-lymphocyte number (Kehrl *et al.*, 1986), and the secretion of IFN-γ leading to a decreased Th1 response to ovalbumin stimulation (Bellone *et al.*, 1995). In addition, it has been identified that Th1 cells can inhibit Th2 cell formation by suppressing interleukin-4 secretion (Gajewski and Fitch, 1988). Indeed, a lack of inhibition of Th2 by Th1

may account for the increased Th2 response in heifers on treatment H (Spellberg and Edwards, 2001).

5.4.3 Hepatic mineral concentration and function enzymes

The liver is generally thought to be the primary organ of Cu storage (Laven and Livesey, 2005), and one of the first biochemical changes to occur when dietary Cu is supplied above the animal's requirements is an increase in hepatic Cu concentration (Suttle, 2010). The ability of Cu supplied in the form of an intra-ruminal CuO bolus to increase hepatic Cu concentrations has already been discussed (Yost *et al.*, 2002), and so it is not surprising, that heifers on treatment H had higher hepatic Cu concentrations throughout the duration of the study. It was however surprising that there were very high hepatic Cu concentrations for heifers on either treatment at 7 months of age in the current study, with concentrations of 798 and 643 mg Cu/kg DM for those on H and C respectively. Hepatic Cu concentrations of this magnitude are well in excess of the 508 mg/kg DM considered to put animals at risk of developing clinical symptoms of Cu toxicity (Livesey *et al.*, 2002). Indeed, the accumulation of Cu at toxic concentrations within the liver can result in damage to hepatocytes with a subsequent release of enzymes involved in liver function into the bloodstream (Giannini *et al.*, 2005). High hepatic Cu concentrations at 7 months of age were subsequently associated with high serum concentrations of GLDH, coupled with high plasma concentrations of GGT, indicative that heifers on both treatments were subject to considerable liver damage at the beginning of the study (Johnston *et al.*, 2014). Lower hepatic Cu concentrations of 350 and 155 mg/kg DM at 13 months of age were observed for heifers on H and C in the current study, which were associated with a decline in GLDH and GGT values to within their normal range (Bidewell *et al.*, 2012). The elevated hepatic Cu concentrations early in the study were most likely caused by heifers receiving the pelleted concentrate *ad-libitum* prior to commencing the study, which had a mean Cu concentration that was approximately 3.0 times the heifers predicted requirements (ARC, 1980; NRC, 2001). The effects of this high dietary concentration may have been further compounded as it was supplied to these animals at a time when mature rumen function was potentially still under development and apparent Cu absorption would have been higher (Bremner and Dalgarno, 1973). Indeed, Suttle (1975) reported a decrease in apparent Cu availability from 74.2% pre-weaning to 10.8% post-weaning in artificially reared lambs.

The difference in hepatic Cu concentrations between the two treatments remained relatively stable between 13 months of age and 6 weeks pre-calving, despite heifers on treatment H

receiving a dietary Cu supply that was approximately 2.7 times the maximum nutritional guideline (ARC, 1980; Table 2.4). The current study is not alone in reporting a plateau in hepatic Cu concentration when dietary Cu is supplied to cattle above requirements (Arthington, 2005; Stoszek *et al.*, 1986). Yost *et al.* (2002) reported a plateau in the hepatic Cu accumulation of Holstein heifers at day 28 following the administration of a 25 g CuO intra-ruminal bolus supplement to a basal ration containing 4.81 mg Cu/kg DM. The precise mechanisms for this apparent adjustment to dietary Cu supply remain unclear (Yost *et al.*, 2002), however it is thought that a Cu absorption blocking and/or excretory mechanism may exist to prevent toxic Cu over-loading of the liver (Stoszek *et al.*, 1986). Evidence for these mechanisms in ruminants is scarce (Suttle, 2010), but there is a suggestion that ATP7B can translocate from the golgi apparatus into the bile canalicular membrane and increase biliary Cu excretion in response to excess dietary Cu (Hernandez *et al.*, 2008).

5.5 Conclusions

Increasing dietary Cu concentrations above requirements increased growth rate and body condition of Holstein Friesian heifers. Increased dietary Cu concentrations however decreased conception rates, despite achieving puberty earlier and at a lower live weight. Reasons for these differences in performance when heifers are fed dietary Cu concentrations in excess of requirements are not clear and require further investigation but may relate to IGF-1. Dietary Cu concentration also modulated the immune response, however deciding which is the most important component of the immune system with respect to long term performance and health is not currently possible. This is an issue that needs addressing before the modulatory effects of Cu upon immune function can be considered either positive or negative.

CHAPTER 6: Effect of periparturient and early lactation Cu supplementation on replacement Holstein-Friesian heifer performance, health and indicators of Cu status.

6.1 Introduction

Interest in the addition of copper (Cu) to ruminant diets centres around its role as an essential trace element required for the production of numerous Cu-dependent enzymes including ceruloplasmin, lysyl oxidase, tyrosinase, and cytochrome *c* oxidase (Suttle, 2010). Clinical Cu deficiency in ruminants presents with symptoms such as anaemia (Suttle *et al.*, 1987), and the impairment of various bodily functions including reproduction, growth, and connective tissue development (Mackenzie *et al.*, 2001; McDowell, 1985; Mills *et al.*, 1976). A deficiency in Cu can be considered either primary, where dietary Cu supply is lacking (Phillippo *et al.*, 1987a), or secondary, where interactions with other elements such as Fe, S or Mo, inhibit Cu absorption and/or function (Phillippo *et al.*, 1987b). Secondary deficiency is generally thought more common and economically important (Suttle, 2010), however the preventative measure undertaken in either instance is to increase dietary Cu supply (Suttle and McLauchlin, 1976). There is however recent evidence to suggest over-supplementation of Cu to cows within the UK industry (Kendall *et al.*, 2015). In a mineral survey of the UK's winter-fed dairy cows in Central and Northern England, Sinclair and Atkins (2015) reported that 32 out of 50 farms were feeding above the then current guideline industry maximum of 20 mg Cu/kg DM (ACAF, 2010). Indeed, excessive dietary Cu supply on farm may also have consequences for food supply chain safety (Fukuda *et al.*, 2004). Kendall *et al.* (2015) monitored liver Cu concentrations in cull cattle passing through a British abattoir over a period of three days, and reported that 65% of Holstein-Friesian cull cows had either toxic or high liver Cu concentrations. This issue may not be confined to the UK, as Castillo *et al.* (2013) reported that Cu was being provided at 1.9 times above NRC (2001) recommendations on Californian dairy farms, with 4 of the 39 farms surveyed providing Cu in excess of three times the recommended dietary Cu concentration (NRC, 2001; Castillo *et al.*, 2013). It has been suggested that excess dietary Cu provision may result from the perception by some in the industry, that in the absence of clinical toxicity, there is no detrimental effect of supplementation above requirement on health or performance (Kendall *et al.*, 2015), and so British dairy herds have continued to over-supply Cu (Jacklin, 2016). Evidence is however growing to suggest harmful sub-clinical effects of over-supplementation of Cu, including increased calf mortality and impaired rumen function (Arthington, 2005; Hunter *et al.*, 2013) in conjunction with anecdotal evidence from

practising vets of an association between altered immune status and elevated hepatic Cu concentrations (Howie, 2017). Indeed, a modulation of the immune response was observed, and conception rates were decreased, despite improved growth rates, when Holstein-Friesian heifers between 4 and 22 months of age in Chapter 5 received dietary Cu concentrations that were approximately 2.5 times their predicted requirements (ARC, 1980; NRC, 2001). Studies examining the effects of Cu over-supplementation on lactating dairy cattle have tended to be short-term in duration (Chase *et al.*, 2000), and have neglected the potential secondary effects that Cu supplementation during the rearing phase may have on the ensuing lactation (Engle *et al*, 2001). There is subsequently a need to determine the effect of periparturient and early lactation Cu supplementation to replacement Holstein-Friesian heifers that have been reared on either a recommended or higher dietary Cu concentration upon their performance, health, fertility, and welfare.

6.2 Materials and Methods

6.2.1 Animals, treatments, housing and management

This study was undertaken at Harper Adams University and all procedures involving animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 (Amended 2013). Twenty-four Holstein Friesian heifers from the original eighty described in Chapter 5 were used from 6-weeks prepartum when they were 24 months of age (SE ± 0.4), and had a mean live weight of 600 kg (SE ± 9.7). These animals commenced the study between mid-June and mid-September 2018, and remained on the study until 14 weeks post-calving (98 days). Heifers were not oestrus synchronised in chapter 5 to avoid any potential confounding effects that this may have had on fertility measurements (Rivera *et al.*, 2004). This resulted in a large calving spread with heifers on either treatment calving between March 2018 and February 2019. In order to balance the environmental conditions between the two treatments (Lucas, 1960), heifers were only recruited on to the lactation study if they calved within a maximum of 7 days of a corresponding heifer on the other treatment, who also entered the study (Lucas, 1960).

Heifers continued to receive the original levels of Cu supplementation (Control vs. High; C vs. H) to which they were assigned in Chapter 5. The dry and lactating control diets were formulated to contain approximately 15 mg of Cu/kg DM estimated to meet the animal's requirement (ARC, 1980; NRC, 2001; Table 6.1). Heifers on the high level of supplementation continued to receive additional Cu in the form of two intra-ruminal Cu oxide

boluses (Agrimin, Ltd, Kirmington, UK) administered on a 6-monthly basis as described in Chapter 5. Calving date was determined by conception date, whereas the date of a new bolus administration was determined by the date on which a heifer commenced the study in Chapter 5. This meant that calving date dependent, heifers on H were bolused either at the beginning, middle or end of the lactation study as determined by their previous bolus date. A bolus size of 16.55 g of CuO was used which predicted to supply 160 mg of Cu/d throughout the study. Based on the predicted DM intake throughout the 6-week dry period (Hoffman *et al.*, 2008; Equation 6.1), and the 14 weeks of lactation (NRC, 2001; Equation 6.2), heifers on treatment H were predicted to receive an additional 10 (9.7) mg Cu/kg DM to provide a total dietary Cu concentration of approximately 25 mg of Cu/kg DM throughout the duration of the study. This concentration for H was similar to the mean concentration of 28 mg Cu/kg DM fed to early lactation dairy cows during the winter-feeding period within the UK (Sinclair and Atkins, 2015).

During the 6-week periparturient period heifers were housed on straw, in the same area of an open span building, and received a straw based dry cow TMR until parturition (Table 6.1). Postpartum, cows transitioned into free stalls in the same area of an open span building fitted with foam mattresses, and were offered a lactation TMR at 105% of *ad-libitum* intake (Table 6.1). The free stalls were bedded three times per week on a Monday, Wednesday, and Friday with a lime-sawdust mix. The dietary components were mixed using a Hi-spec forage mixer calibrated to ± 0.1 kg, and the lactation TMR was fed via roughage intake feeders (Hokofarm, Marknesse, Netherlands), fitted with an automatic animal identification and weighing system calibrated to ± 0.1 kg (Sinclair *et al.*, 2007), with refusals collected prior to feeding three times per week on a Monday, Wednesday, and Friday. Animals had continual access to fresh water that contained S, Cu, Fe, and Mo concentrations of 22.2 mg/L, 1.6 µg/L, 2.8 µg/L, 0.13 µg/L respectively throughout the duration of the study.

Table 6.1. Ingredient (g/kg DM) composition of the dry cow TMR offered to cows during the 6-week prepartum period, and the lactation TMR offered during lactation.

Item, g/kg DM	Dry cow	Lactation
Chopped wheat straw	474	19
Maize silage	151	354
Lucerne silage	104	165
Proflo syrup[1]	142	--
Sweet starch[2]	--	94
Soy hulls	--	94
Rapeseed meal	34	85
Wheat distillers dark grains	34	85
Soy bean meal[3]	14	36
Dry cow minerals[4]	13	--
Magnesium chloride	12	--
Palm kernel meal	9	24
Rumen protected fat[5]	--	16
Provimi lift[6]	6	--
Lactating cow minerals[7]	--	7
Buffer[8]	--	5
Limestone flour	4	4
Salt	--	4
Molasses	3	7
Saccharomyces cerevisae[9]	--	1

[1] Wheat Vinasses, KW Alternative Feeds, Andover, UK. 12.5 mg of Cu/kg DM declared.
[2] KW Alternative Feeds, Andover, UK (g/kg); 360 cake products, 140 breakfast cereals, 140 cocoa hulls, 140 wheat feed, 70 sugar confectionary, 140 flour.
[3] Rumen protected soya bean meal, KW Alternative Feeds, UK.
[4] Dry cow mineral/vitamin premix, KW Alternative Feeds, Andover, UK. Major minerals (g/kg): Ca 50, P 40, Mg 200, Na 50; Trace minerals (mg/kg): Cu 757, Se 40, Zn 6,000, Mn 4,000, I 400, Co 80; vitamins (iu/kg) were: Vitamin A 800,000, vitamin D 200,000, and vitamin E 10,000.
[5] Megalac, Volac International Ltd, Royston, UK.
[6] Provimi lift, Cargill, PLC, Surrey, UK.
[7] Lactating cow mineral/vitamin premix, KW Alternative Feeds, Andover, UK. Major minerals (g/kg): Ca 220, P 30, Mg 80, Na 80; Trace minerals (mg/kg): Cu 1000, Se 40, Zn 6,000, Mn 4,000, I 400, Co 80; vitamins (iu/kg) were: Vitamin A 1,000,000, vitamin D 300,000, and vitamin E 4,000.
[8] Acid buf, Celtic Sea Minerals, Cork, Ireland.
[9] SC digestaid, Biotal, Worcestershire, UK.

Table 6.2. Chemical composition of the dry cow TMR offered to cows during the 6-week prepartum period, and the lactation TMR offered during lactation.

Chemical composition	Dry cow	Lactation
DM, g/kg	472	507
Crude protein, g/kg DM	132	167
Ash, g/kg DM	80	78
Organic matter, g/kg DM	920	922
NDF, g/kg DM	551	366
ADF, g/kg DM	358	270
Ether extract, g/kg DM	19	48
Starch, g/kg DM	59	180
WSC, g/kg DM	27	48
Gross energy, MJ/kg of DM	18.0[1]	19.0
Metabolisable energy, MJ/kg of DM	9.6[1]	11.7[1]
Ca, g/kg DM	7.13	10.44
P, g/kg DM	3.42	3.88
Mg, g/kg DM	4.67	2.32
S, g/kg DM	2.01	2.10
Cu, mg/kg DM	14.4	14.5
Mo, mg/kg DM	1.1	1.4
Zn, mg/kg DM	120.9	90.2
Fe, mg/kg DM	223	189
Mn, mg/kg DM	100.3	81.1

[1] Calculated using standard and declared values (Andrew, 1991; KW Alternative Feeds, 2019; MAFF, 1982; Sauvant *et al.*, 2002).
[2] Based on predicted intakes during the dry period (mean = 10.7 kg DM/d), and measured intakes during lactation, cows on treatment H were supplemented at a mean of 8.8 mg Cu/kg DM throughout the study to provide a total dietary concentration of 23.2 mg/kg DM.

6.2.2 Experimental routine

The cows were weighed and condition scored (Ferguson *et al.*, 1994), at housing (6-weeks preparturition), within 6 hours post-partum, and on a weekly basis thereafter. Animals had their wither height measured within 6 hours postpartum, and again at the end of the study (Week 14). Wither height was determined using a height measuring stick in a procedure adapted from Jeffery and Berg (1972). Cows were milked twice daily through a Westaflia 40-point internal rotary parlour at approximately 1600 and 0600 h. The milk yield of each cow was recorded at each individual milking, whilst samples were taken fortnightly at consecutive afternoon and morning milkings for subsequent analysis of somatic cell count (SCC) and milk composition. Fresh samples of the dry and lactating diets were collected on a weekly basis immediately post-feeding and stored at -20 ^{o}C prior to subsequent proximate and mineral analysis.

Blood samples were collected by jugular venepuncture at 1100 h during weeks -6 (six-weeks prepartum), 0 (within 6 hours postpartum), and 1, 2, 4, 8, and 14 postpartum into vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK) containing silica gel (to determine ceruloplasmin and glutamate dehydrogenase; Cp and GLDH), lithium heparin (to determine β-hydroxybutyrate and non-esterified fatty acids; BHB and NEFA), fluoride/oxalate (to determine glucose), dipotassium ethylenediaminetetraacetic acid (K_2EDTA; to determine the haematological profile), and potassium ethylenediaminetetraacetic acid (K_3EDTA; to determine plasma mineral concentrations). Liver biopsy samples were collected via the 11th intercostal space as described by Davies and Jebbet (1981) at weeks -6 and 14, frozen in liquid nitrogen, and stored at -80 ^{o}C prior to subsequent analysis.

The humoral immune response of the cows was assessed using keyhole limpet haemocyanin (KLH) according to Mackenzie *et al.* (1997a). Cows were challenged during week 4 of the study, they were immunised at approximately 1100 h subcutaneously over the 5th rib on the right side using 1 mL of 1 mg/mL KLH (Sigma-Aldrich, Dorset, UK) precipitated on alum (Pollock *et al.*,1991). Blood samples were then collected at approximately 1100 h on days 0 (Wk 4), 7 (Wk 5), 14 (Wk 6), 21 (Wk 7), 28 (Wk 8), and 63 (Wk 13), into vacutainers (Becton Dickson Vacutainer Systems, Plymouth, UK) containing silica gel, with the resulting serum stored at -20 ^{o}C prior to chemical analysis (Mackenzie *et al.*, 1997a). Postpartum, cows received conventional (non-sexed) Holstein or British Blue semen by artificial insemination (AI) following a voluntary waiting period of approximately 50 days. IceQube (IceRobotics Ltd, Edinburgh, UK) pedometers were attached to the back-left leg of each cow in order to detect oestrus. Additional replicates (n = 23 for L, and n = 29 for H) were used for all fertility measurements which included conception rate to 1st service, and 100 day in calf rate (proportion of cows in calf at 100 days). These cows received the same diets and treatments as described in Section 6.2.1. Twenty-four (n = 15 for L and n = 9 for H) of the original 80 heifers were removed from the study during an out-wintering period on kale after completion of the growing study (Chapter 5).

6.2.3 Chemical analysis

Weekly dry cow and lactation TMR samples were bulked on a bi-monthly basis and analysed according to AOAC (2012) for dry matter (DM; 934.01; intra-assay CV of 1.4%), crude protein (CP; 990.03; Section 3.1.1; intra-assay CV of 1.7%), ash (942.05; Section 3.1.2; intra-assay CV of 1.2%), and ether extract (EE; 2003.05; Section 3.1.5; intra-assay

CV of 3.6%). These samples were also analysed for starch (Section 3.1.6; intra-assay of 2.9%) by polarimetric method as described by ISO 6493 (2000) at Sciantic Analytical (Stockbridge Technology Centre, North Yorkshire, UK). The gross energy (GE) content of the lactation TMR was determined using a bomb calorimeter (Parr 6200; Parr Instruments Company, Illinois; intra-assay CV of 1.5%). The acid detergent fibre (ADF; Section 3.1.4; intra-assay CV of 3.0%) and neutral detergent fibre (NDF; Section 3.1.4; intra-assay CV of 2.5%) content of the feeds was determined according to Van Soest *et al.* (1991), heat-stable α-amylase was used in the determination of NDF (Sigma-Aldrich, Dorset, UK). In addition, the water-soluble carbohydrate (WSC; Section 3.1.7) content of the samples was determined according to Thomas (1977; intra-assay CV of 3.2%). The macro and micro mineral content of the bulked feed samples was analysed by inductively coupled plasma-mass spectrometry (ICP-MS; Nexion 2000; Perkin Elmer, Beaconsfield, UK; Section 3.1.8), following digestion and extraction using a DigiPrep system (QMX Laboratories, Essex, UK) as described by Cope *et al.* (2009).

Plasma samples were analysed for glucose, BHB and NEFA (Randox Laboratories, Antrim, UK; kit catalogue no. GL1611, RB1008, and FA115; Section 3.2.5; intra-assay CV of 1.2%, 4.8% and 1.0% respectively). Serum samples were analysed for GLDH (Randox Laboratories, Antrim, UK; kit catalogue no. GL441; Section 3.2.5; intra-assay CV of 2.3%) and Cp according to Henry *et al.* (1974; Section 3.2.3; intra-assay CV of 1.6%), and whole blood was analysed for SOD activity (Randox Laboratories, Antrim, UK; kit catalogue no. SD125; Section 3.2.4; intra-assay CV of 3.8%). The analysis of serum, plasma, and whole blood parameters was conducted using a Cobas Miras Plus auto-analyser (ABX Diagnostics, Bedfordshire, UK). Haematological parameters including white blood cell counts, red blood cell counts, haematocrit, and haemoglobin were determined using a Vet Animal Blood Counter (Woodley Equipment Company Ltd, Bolton, UK; Section 3.2.2). Liver and plasma samples were analysed for Cu, Mo, Zn, and Fe (Nexion 2000; Perkin Elmer, Beaconsfield, UK; Sections 3.3 and 3.2.6; intra-assay CV of 0.7%, 1.8%, 1.8%, and 0.8% for liver, and 0.6%, 1.7%, 2.3%, and 2.4% for plasma respectively) in a method adapted from Cope *et al.* (2009). Serum from the KLH immune challenge was analysed for anti-KLH antibody subclasses immunoglobulin G_1 (IgG_1) and IgG_2 according to Mackenzie *et al.* (1997a), by direct ELISA using mouse anti-bovine IgG_1 and IgG_2 monoclonal antibodies (Bio-rad Laboratories, Hertfordshire, UK), and goat anti-mouse IgG_2 conjugated to alkaline phosphatase (Bio-rad Laboratories, Hertfordshire, UK).

6.2.4 Calculations and statistical analysis

Dry matter intakes (kg DM/d) during the prepartum period were predicted according to Hoffman *et al.* (2008) using the equation:

$$\text{DMI (kg/d)} = 15.79 \times \left[1 - e^{(-0.00210 \times \text{BW})}\right] - 0.0820 \times \text{NDFdv} \qquad \text{Equation 6.1}$$

Where, $\text{NDFv} = (\text{dietary NDF as a \% of DM}) - \{22.07 + [0.08714 \times \text{BW}] - [0.00007383 \times (\text{BW})^2]\}$, and BW = live weight (kg). Intakes during the postpartum period were predicted according to NRC (2001) using the equation:

$$\text{DMI (kg/d)} = (0.372 \times \text{FCM} \times BW^{0.75}) \times \left\{1 - e^{|-0.192 \times (WOL+3.67)|}\right\} \qquad \text{Equation 6.2}$$

Where, FCM = 4 % fat corrected milk yield (kg/d), BW = live weight (kg), and WOL = week of lactation. In contrast, the contribution of the CuO boluses to the total dietary Cu concentration (mg/kg DM) presented in the results section (Section 6.3), was calculated using Hoffman *et al.* (2008) during the prepartum period, and measured intakes during the lactation period. The energy content of the milk (MJ/kg) was calculated according to Tyrrel and Reid (1965) using the equation:

$$\text{Milk energy (MJ/kg)} = (\text{F} \times 0.0384) + (\text{P} \times 0.0223) + (\text{L} \times 0.0199) - 0.108$$

Equation 6.3

Where, F = milk fat concentration (g/kg), P = milk protein concentration (g/kg), and L = milk lactose concentration (g/kg). The mean daily ME balance (MEb; MJ/cow/d) for each cow was calculated according to Thomas (2004) using the equation:

$$\text{MEb (MJ/cow/d)} = \left[(\text{M}_{\text{Ml}} \times \text{LW}^{0.75}) + \left(\frac{0.0013 \times \text{LW}}{\text{K}_\text{m}}\right) - 10\right] - \text{Mei}$$

Equation 6.4

Where, M_{ml} = is the ME required for maintenance and milk production (MJ/kg of metabolic live weight), $LW^{0.75}$ = metabolic live weight, K_m = the efficiency of ME utilisation (calculated as 0.35 x ME/gross energy + 0.503), and MEi = ME intake (MJ/cow/d).

Continuous parameters were analysed using repeated measures analysis of variance. Milk SCC underwent a $\log_{10}$ transformation before analysis. The model included the main effects of dietary Cu concentration, time, their interaction, and was analysed as:

$$Y_{ijkl} = \mu + B_i + C_j + T_k + F.T_{jk} + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of Cu concentration (j = C vs. H); T_k = effect of time; $F.T_{jk}$ = interaction between Cu concentration and time, and ε_{ijkl} = residual error.

Binomial data (e.g. 100 day in calf rate) was analysed using the logit function using linear model regression analysis. Significance was identified using chi-squared, whilst the model included treatment (Cu concentration) as a term. Non-continuous performance and blood parameters including liver mineral concentrations were analysed by analysis of variance as:

$$Y_{ijkl} = \mu + B_i + C_j + \varepsilon_{ijk}$$

where Y_{ijk} = dependent variable; μ = overall mean; B_i = fixed effect of blocks; C_j = effect of Cu concentration (j = C vs. H), and ε_{ijkl} = residual error. All statistical analyses were undertaken using Genstat version 18 (VSN International, Ltd, Oxford, UK), means are presented with their associated 95 % confidence interval or standard error of the difference of the mean where appropriate. $P < 0.05$ was considered as significant, and a trend was considered where $P < 0.1$.

6.3 Results

6.3.1 Dietary analysis, animal performance and fertility

The dry and lactating cow diets had DM contents of 472 and 507 g/kg (Table 6.2), with crude protein and ether extract contents of 132 and 19 g/kg DM for the dry cow diet, compared to 167 and 48 g/kg DM for the lactating cow diet respectively. The lactating cow diet also had a higher starch content (180 vs. 59 g/kg DM) and a lower NDF content (366 vs. 551 g/kg DM) compared to the dry cow diet. The lactating cow diet had a macro mineral content of 10.44, 3.88, 2.32, and 2.10 g/kg DM for Ca, P, Mg, and S, whereas the dry cow diet contained 7.13, 3.42, 4.67, and 2.01 g/kg DM for Ca, P, Mg, and S respectively. Mean Cu concentrations were 14.5 and 14.4 mg/kg DM for the lactating and dry cow diets respectively, resulting in a mean dietary Cu concentration of 14.5 mg/kg DM for the basal ration throughout the duration of the study. The CuO boluses were calculated to supply an additional 8.8 mg Cu/kg DM, resulting in a total dietary concentration of 23.2 mg Cu/kg DM for cows on H over the duration of the study. The lactation diet contained higher concentrations of S and Mo of 2.10 g S/kg DM and 1.4 mg Mo/kg DM, compared to 2.01 g S/kg DM and 1.1 mg Mo/kg DM for the dry cow diet respectively. In contrast, the dry cow diet contained a higher Fe concentration of 223 mg/kg DM compared to 189 mg/kg DM for

the lactation diet. Mean basal dietary S, Mo, and Fe concentrations over the duration of the study were subsequently 2.07 g/kg DM, 1.3, and 199 mg/kg DM respectively.

Table 6.3. Housing (6-weeks prepartum) and postpartum performance of primiparous cows fed either a control or a high dietary copper concentration from 4 months of age until week 14 of lactation.

	Cu supplementation level				
	C	H	SED	CI (95%)	P-value
Live weight at housing, kg	597	602	19.8		0.784
Parturition live weight, kg	592	602	13.5		0.582
Final live weight, kg	597	612	14.4		0.300
Δ live weight[1], kg/d	0.22	0.31	0.094		0.365
Condition score at housing	2.85	3.18	0.084		0.004
Parturition condition score	2.93	3.20	0.079		0.007
Final condition score	2.93	2.98	0.097		0.619
Δ condition score[1]	0.00	-0.23	0.079		0.019
Parturition wither height, cm	144	142	1.4		0.208
Final wither height, cm	146	143	1.6		0.182
DMI, kg/d	21.2	21.7	0.69		0.491
ME intake, MJ/cow/d	248	254	46.3		0.491
Milk yield, kg/d	33.1	32.0	1.58		0.518
Fat, g/kg	42.1	42.2	1.42		0.975
Protein, g/kg	32.5	31.8	0.70		0.350
Lactose, g/kg	46.3	46.9	0.42		0.155
Urea, mg/dL	22.8	19.3	0.80		0.006
Fat yield, kg/d	1.33	1.35	0.090		0.826
Protein yield, kg/d	1.08	1.02	0.035		0.241
Lactose yield, kg/d	1.56	1.51	0.076		0.741
Milk SCC, $\log_{10}$/mL	1.66	1.48	0.171		0.300
Milk energy, MJ/kg	3.15	3.16	0.064		0.838
Milk energy output, MJ/cow/d	104	101	4.7		0.535
ME balance, MJ/cow/d	-5	-20	5.9		0.019
Pregnancy to 1st service, %	45	45		0.34, 3.12	0.964
100d in-calf rate, %	73	52		0.76, 8.16	0.125

[1] Parturition to Week 14.
[2] Fertility measurements (n = 22 for C; n = 29 for H).

There was no difference (P > 0.05) in cow live weight at housing, parturition or at the end of the study (Table 6.3), although there was an effect (P < 0.001) of time on live weight (Figure 6.1a), which decreased for cows on either treatment between parturition and week 2/3 of lactation, with an increase thereafter. There was also no effect (P > 0.05) of treatment

on live weight gain between parturition and week 14 of lactation. In contrast, cows on treatment H had a higher (P < 0.01) mean condition score at housing (3.18 vs. 2.85) and parturition (3.20 vs. 2.93) but not at the end of study (2.98 vs. 2.93), and subsequently those on H had a greater body condition score loss of -0.23 compared to 0.00 for those on treatment C between parturition and week 14 of lactation. In contrast, there was no effect (P > 0.05) of treatment on mean condition score throughout the duration of the study (Figure 6.1b). Wither height at parturition and at the end of the study were also not affected (P > 0.05) by dietary treatment.

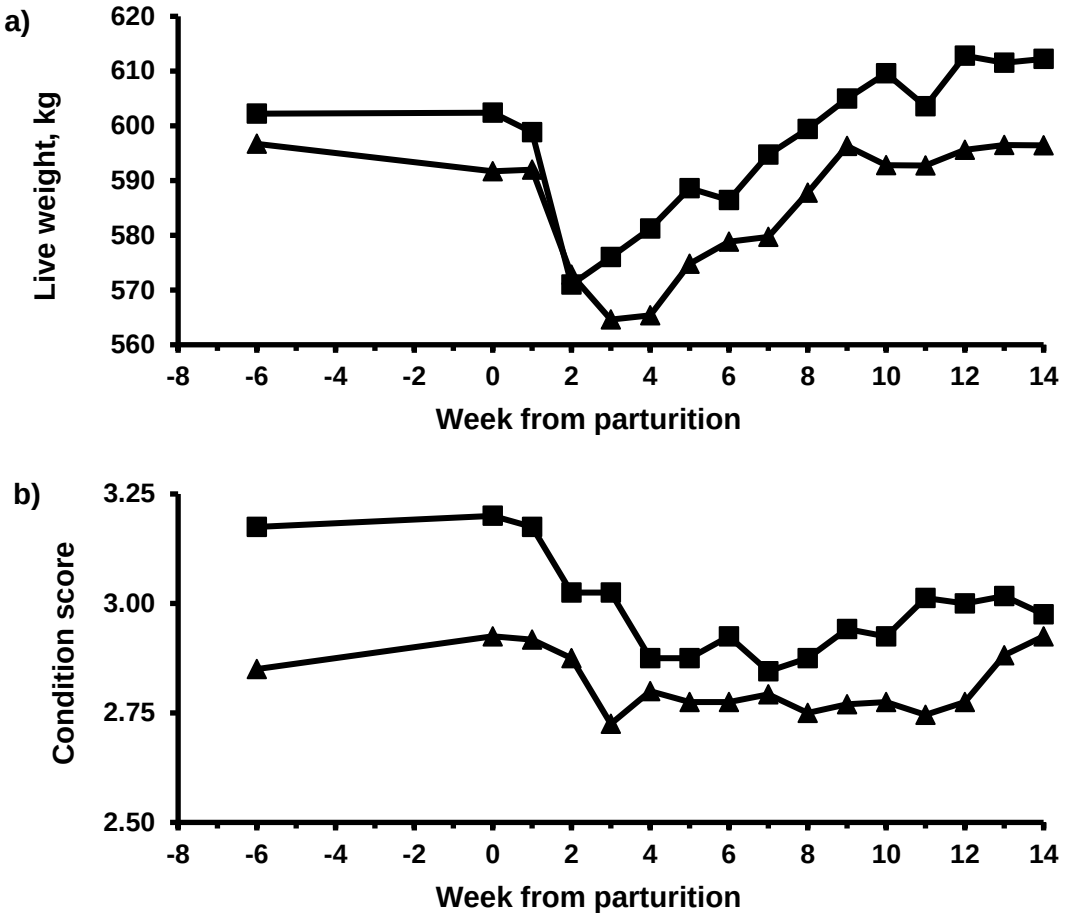

Figure 6.1. Live weight (a) and condition score (b) of primiparous dairy cows from housing until week 14 of lactation (measured weekly post postpartum), that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For live weight; pooled SED = 21.7. Copper, P = 0.593; time, P < 0.001; copper x time, P = 0.657. For condition score; pooled SED = 0.112. Copper, P = 0.103; time, P < 0.001; copper x time, P = 0.089.

There was no effect (P > 0.05) of dietary treatment on mean DMI (21.5 kg/d) which increased (P < 0.001) for cows on either treatment between weeks 2 and 14 of the study (Figure 6.2a). Similarly, there was no effect (P > 0.05) of treatment on milk yield with a mean

value of 32.6 kg/d across both treatments, although there was an effect of time (P < 0.001), where the yield of cows on either treatment increased between weeks 2 and 14 of lactation (Figure 6.2b). There was also a treatment x time interaction (P < 0.001) on milk yield, which was 4.5 kg/d higher at week 2 of lactation for cows on treatment C compared to H.

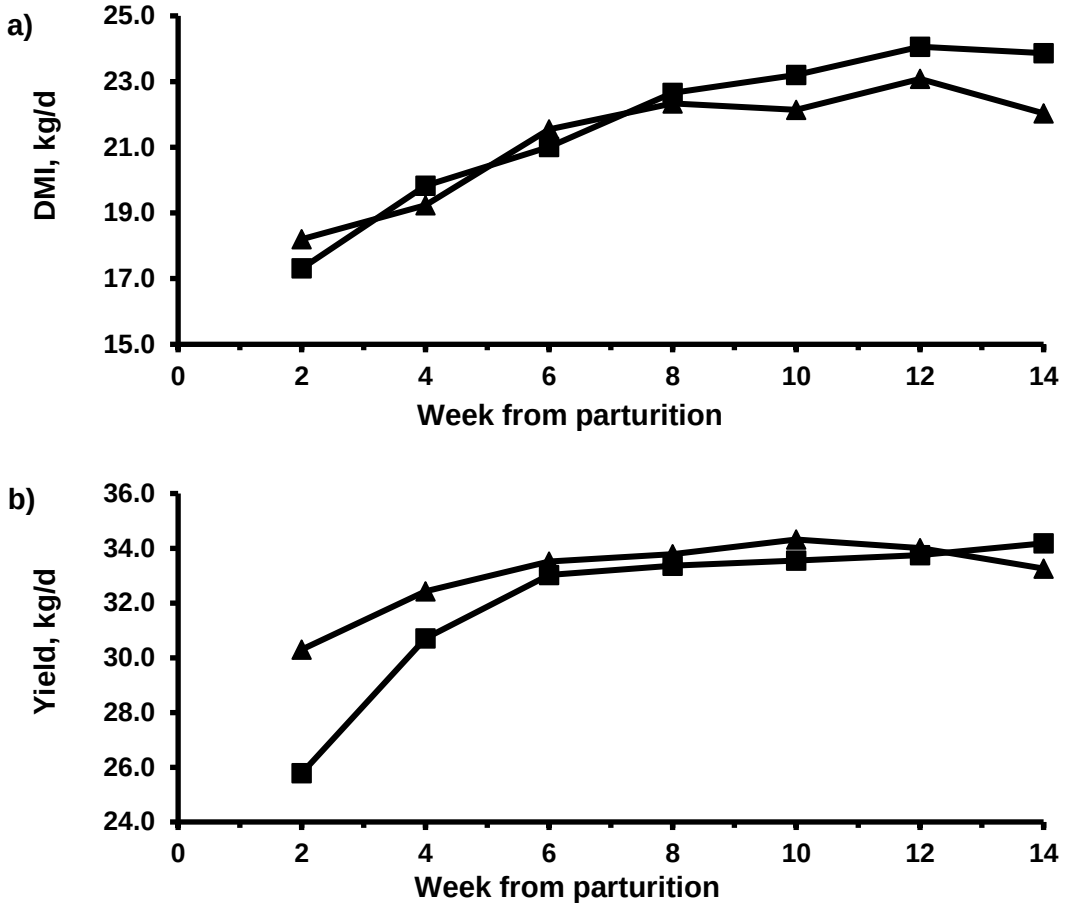

Figure 6.2. Fortnightly DMI (a) and milk yield (b) of primiparous dairy cows during the first 14 weeks of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For DMI; pooled SED = 0.91. Copper = 0.491; time, P < 0.001; copper x time, P = 0.098. For milk yield; pooled SED = 1.70. Copper, P = 0.518; time, P < 0.001; copper x time, P < 0.001.

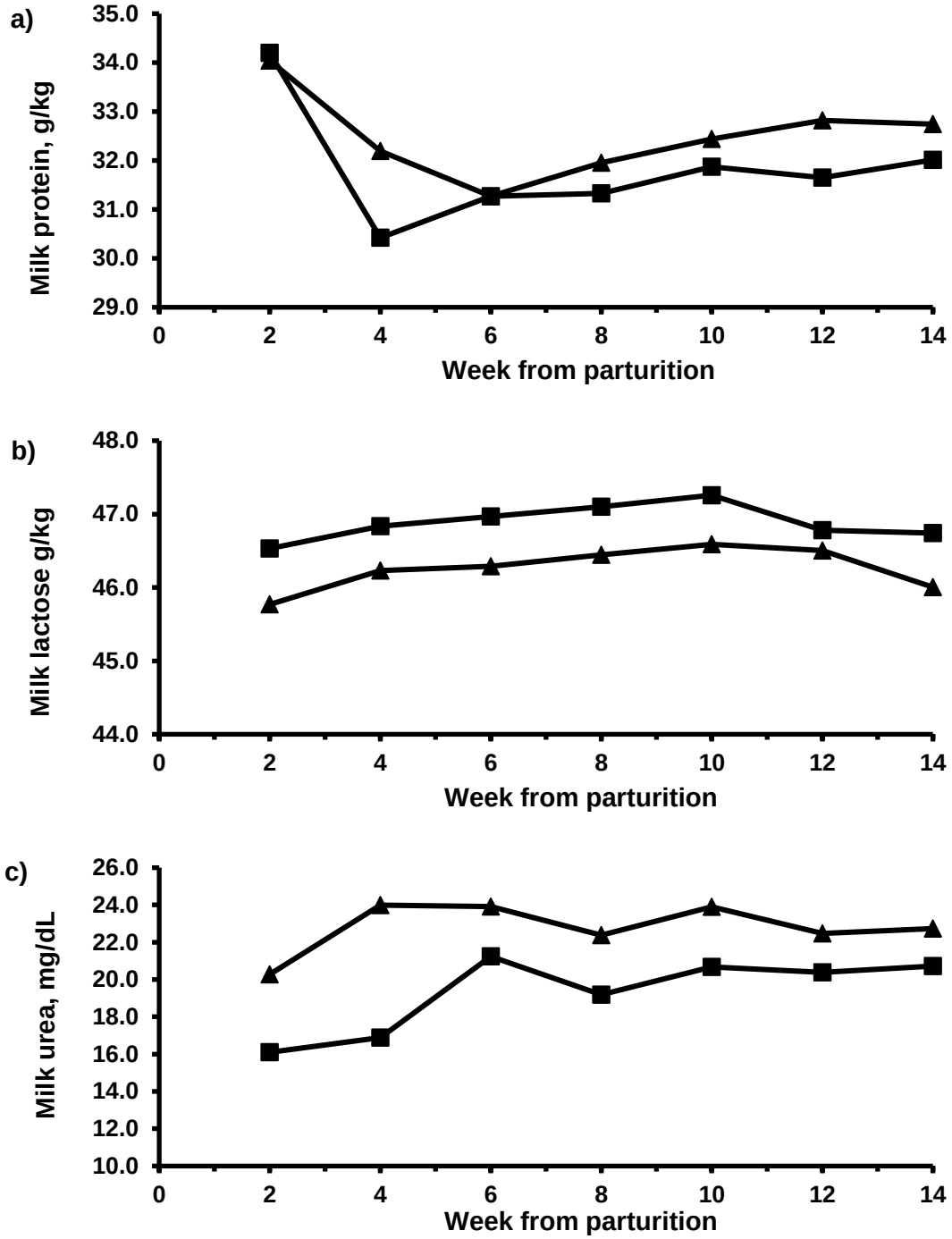

Figure 6.3. Fortnightly milk protein (a), lactose (b), and urea (c) concentrations of primiparous dairy cows during the first 14 weeks of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For protein; pooled SED = 0.97. Copper, P = 0.350; time, P = 0.012; copper x time, P = 0.398. For lactose; pooled SED = 0.49. Copper = 0.155; time, P = 0.036; copper x time, P = 0.757. For urea; pooled SED = 1.32. Copper, P = 0.006; time, P = 0.031; copper x time, P = 0.320.

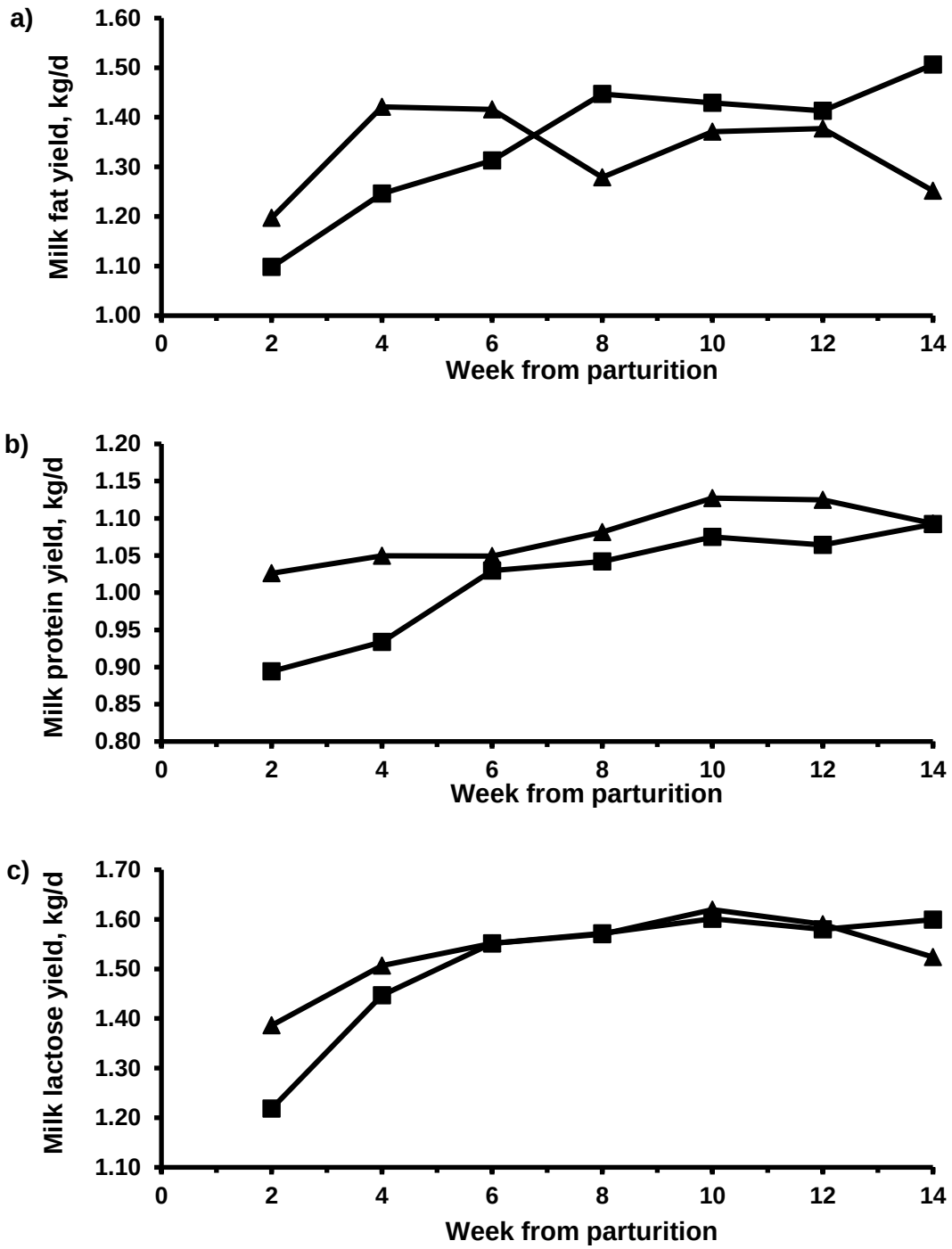

Figure 6.4. Fortnightly milk fat (a), protein (b), and lactose (c) yields of primiparous dairy cows during the first 14 weeks of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For fat; pooled SED = 0.117. Copper, P = 0.826; time, P = 0.013; copper x time, P = 0.031. For protein; pooled SED = 0.056. Copper, P = 0.241; time, P < 0.001; copper x time, P = 0.094. For lactose; pooled SED = 0.082. Copper, P = 0.741; time, P < 0.001; copper x time, P = 0.005.

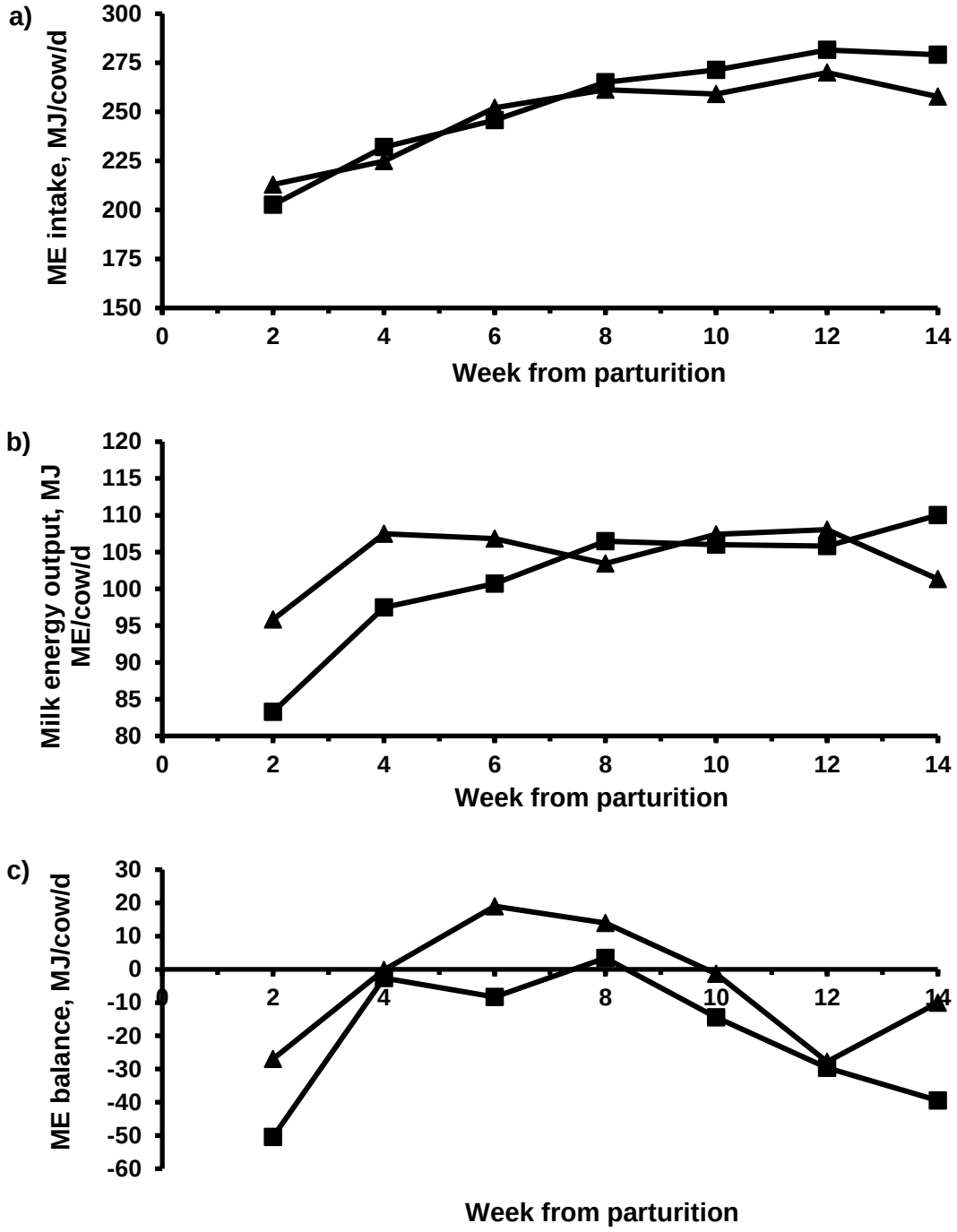

Figure 6.5. Fortnightly metabolisable energy (ME; a) intake, milk energy output (b), and ME balance (c) of primiparous dairy cows during the first 14 weeks of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For ME intake; pooled SED = 10.7. Copper, P = 0.491; time, P < 0.001; copper x time, P = 0.098. For milk energy output; pooled SED = 5.7. Copper, P = 0.535; time, P < 0.001; copper x time, P = 0.012. For ME balance; pooled SED = 12.7. Copper, P = 0.019; time, P = 0.021; copper x time, P = 0.729.

There was no effect ($P > 0.05$) of treatment on milk fat, protein, or lactose concentrations with mean values of 42.1, 32.1, and 46.6 g/kg across the study respectively (Table 6.3). There was however an effect ($P < 0.05$) of time on both milk protein and lactose content, milk protein fluctuated with time (Figure 6.3a), and milk lactose increased for cows on either treatment between week 2 and 14 of lactation (Figure 6.3b). In contrast, cows on treatment C had a 3.5 mg/dL higher ($P < 0.01$) milk urea content than those on H throughout the study (Figure 6.3c). Indeed, there was also an effect ($P < 0.05$) of time on milk urea content which increased for cows on either treatment during lactation. In contrast, there was no effect ($P > 0.05$) of treatment on milk fat, protein and lactose yield with mean values of 1.34, 1.05, and 1.54 respectively. There was however an effect ($P < 0.05$) of time on the yield of milk components, milk fat yield was observed to fluctuate (Figure 6.4a), whereas milk protein and lactose yield increased between week 2 and 4 of lactation (Figure 6.4b,c). There was also a treatment x time ($P < 0.05$) interaction on milk fat and lactose yield, with cows on treatment C having a higher milk protein (0.13 kg/d) and lactose (0.17 kg/d) yield in week 2 of lactation compared to those on H.

Metabolisable energy intake, milk energy content, and milk energy output were not affected ($P > 0.05$) by treatment, with mean values of 251 MJ ME/cow/d, 3.16 MJ/kg, and 103 MJ/cow/d respectively. In contrast, there was an effect of time ($P < 0.001$) on ME intake and milk energy output (Figure 6.5a,b), where both increased for heifers on either treatment during lactation. There was also a treatment x time interaction ($P < 0.05$) on milk energy output, where cows on treatment C had a 13 MJ/cow/day higher milk energy output during week 2 compared to those on treatment H. Cows on treatment C also had a lower ($P < 0.05$) negative energy balance during lactation compared to those on treatment H (-5 vs. -20 MJ ME/cow/d). There was also an effect ($P < 0.05$) of time on ME balance, which increased for cows on either treatment between week 2 and 6/8 of lactation, with a decrease thereafter. In contrast, there was no effect ($P > 0.05$) of treatment on either pregnancy to first service or 100 d in-calf rate, with mean values of 45% and 63% respectively.

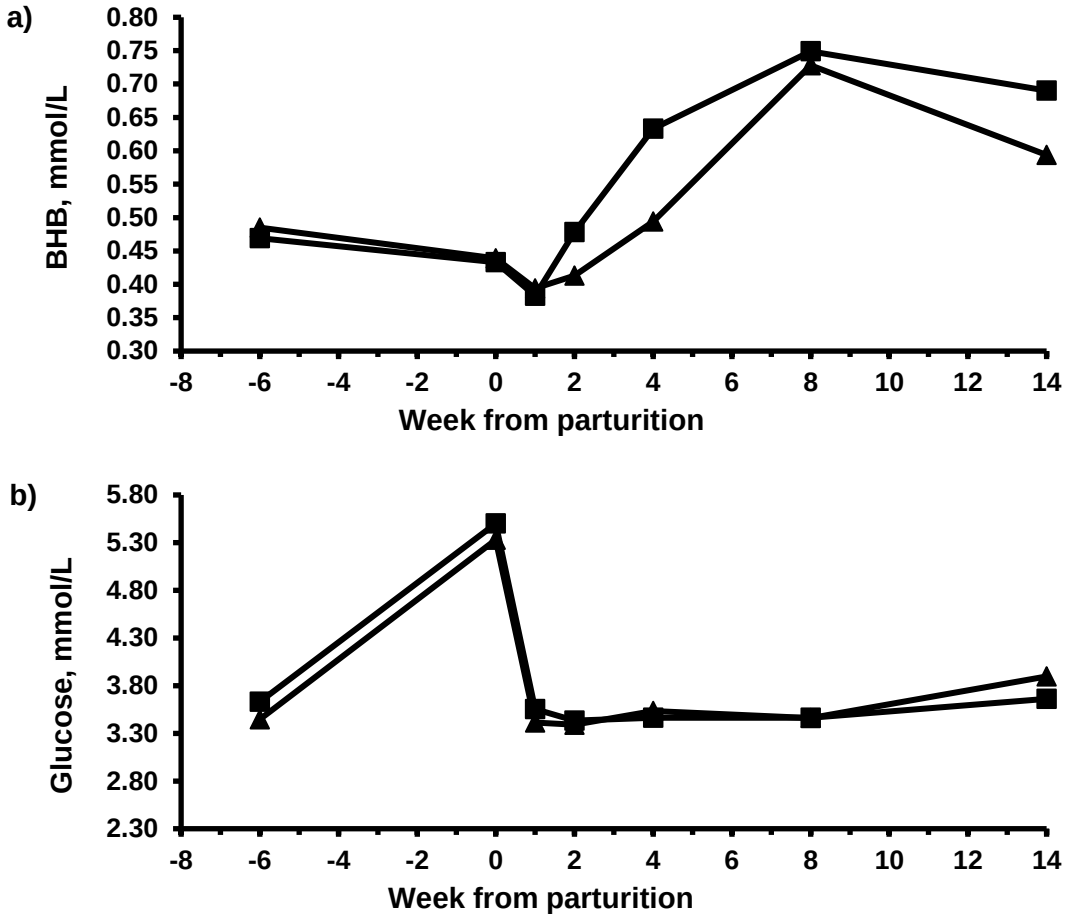

Figure 6.6. Plasma β-hydroxybutyrate (BHB; a) and glucose (b) concentrations of primiparous dairy cows from housing until week 14 of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For plasma BHB; week -6, SED = 0.052, copper, P = 0.763. Week 0, SED = 0.038, copper, P = 0.882. Week 1, SED = 0.036, copper, P = 0.765. Weeks 2-14, pooled SED = 0.083. Copper, P = 0.029; time, P < 0.001; copper x time, P = 0.777. For plasma glucose; week -6, SED = 0.140, copper, P = 0.202. Week 0, SED = 1.020, copper, P = 0.872. Week 1, SED = 0.123, copper, P = 0.272. Weeks 2-14, pooled SED = 0.122. Copper, P = 0.476; time, P < 0.001; copper x time, P = 0.199.

6.3.2 Blood metabolites, plasma mineral concentration, Cu-dependent enzymes, and liver function enzymes

Plasma BHB concentrations were not affected (P > 0.05) by dietary treatment at week -6, 0, and 1 (Figure 6.6a). There was however an effect of treatment (P < 0.05) on plasma BHB concentrations between week 2 and 14 of lactation which were 0.08 mmol/L higher for animals on treatment H compared to those on C. Plasma BHB concentrations were also affected (P < 0.001) by time, where concentrations in cows on either treatment increased from week 2 of lactation to a peak of 0.74 mmol/L at week 8 with a decrease thereafter. In contrast, plasma glucose concentrations were not affected (P > 0.05) by treatment throughout the study with a mean value of 3.54 mmol/L between week 2 and 14 of lactation

(Figure 6.6b). There was however an effect (P < 0.001) of time on plasma glucose concentrations, which increased in cows on either treatment between week 2 and 14 of lactation. Plasma NEFA concentrations were also not affected (P > 0.05) by treatment between week 0 and 14 of the study, with a mean value of 0.37 mmol/L (Figure 6.7a), although values were observed to decrease (P < 0.001) with time from a maximum concentration of 0.68 mmol/L at parturition. In contrast, serum GLDH for cows on treatment H were higher (P < 0.05; 137 vs. 54 U/L) than those on C from week 0 to 14 of lactation (Figure 6.7b). There was also an effect (P < 0.001) of time on serum GLDH concentrations, which increased for cows on either treatment from a mean of 22 U/L at week 0 to a mean of 149 at week 14.

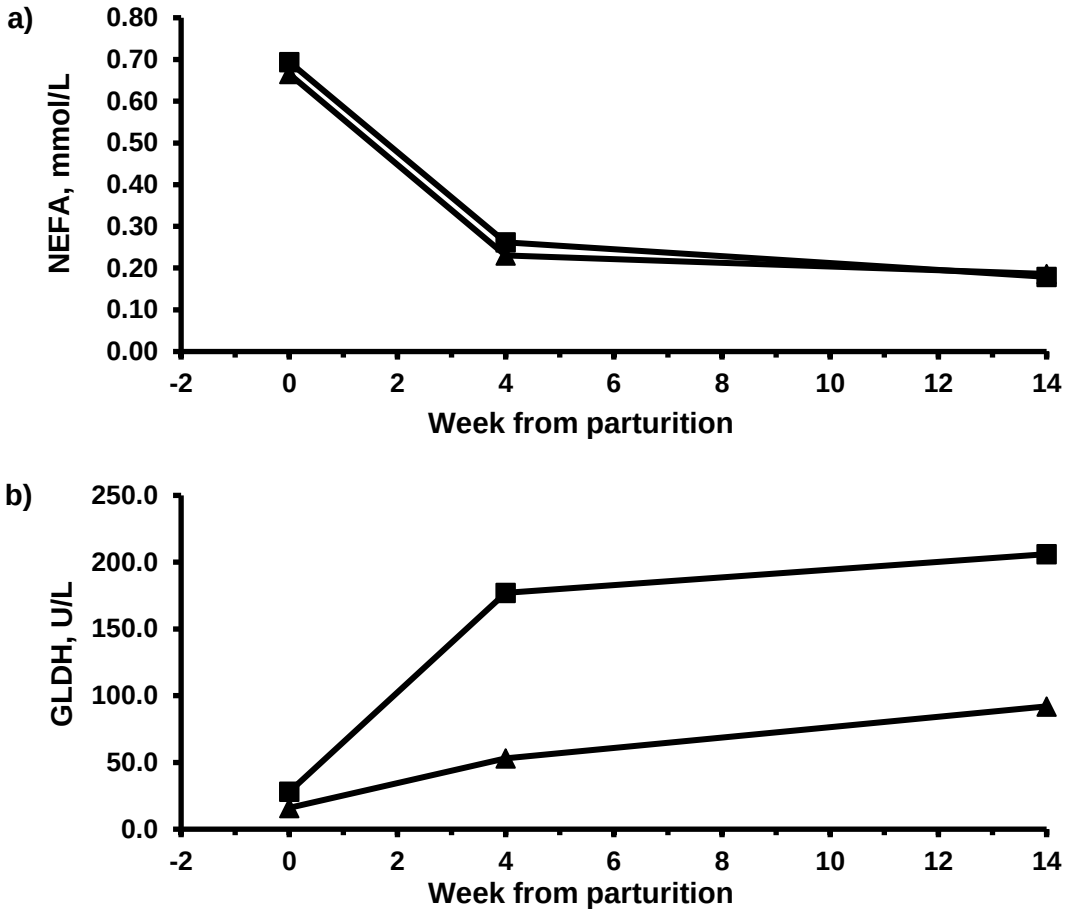

Figure 6.7. Plasma non-esterified fatty acids (NEFA; a) and serum glutamate dehydrogenase (GLDH; b) of primiparous dairy cows during the first 14 weeks of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For NEFA; pooled SED = 0.108. Copper, P = 0.782; time, P < 0.001; copper x time, P = 0.869. For GLDH; pooled SED = 54.70. Copper, P = 0.042, time; P < 0.001; copper x time, P = 0.211.

Table 6.4. Plasma mineral concentrations and Cu-dependent enzymes in primiparous dairy cows from housing until week 14 of lactation, that were fed either a control or a high dietary copper concentration from 4 months of age to week 14 of lactation.

	Cu supplementation level			P-values[1]		
Item[2]	C	H	SED	Cu	T	Cu x T
Plasma Cu, µmol/L	16.5	17.6	0.68	0.167	<0.001	0.294
Plasma Fe, µmol/L	46.2	52.2	4.85	0.236	0.001	0.588
Plasma Zn, µmol/L	22.5	23.6	1.91	0.549	0.005	0.768
Plasma Mo, µmol/L	0.27	0.29	0.050	0.748	0.017	0.378
Serum Cp, mg/dL	20.2	20.3	1.59	0.947	<0.001	0.370
Cp: Cu	1.23	1.16	0.072	0.369	<0.001	0.548
SOD U/g of Hb	3065	2861	272.7	0.463	0.295	0.297

[1] Cu= main effect of Cu source supplementation level, T = effect of time, Cu x T = interaction between supplementation level and time.

[2] Blood samples analysed as follows: plasma mineral concentrations and Cp activity = weeks -6, 0, 1, 2, 4, 8, and 14. SOD activity = 0, 4, and 14.

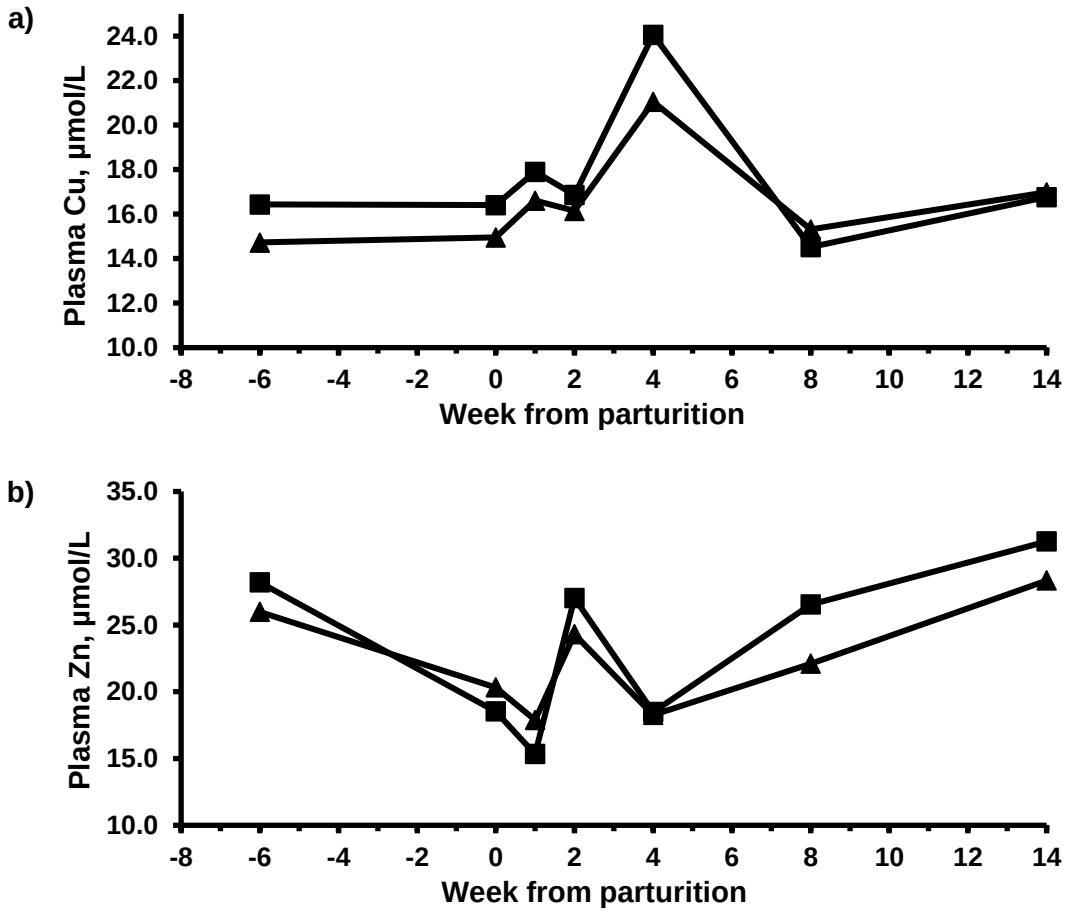

Figure 6.8. Plasma copper (Cu; a) and zinc (Zn; b) concentrations of primiparous dairy cows from housing until week 14 of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For plasma Cu; pooled SED =1.26. Copper, P = 0.167, time; P < 0.001; copper x time, P = 0.294. For plasma Zn; pooled = 4.47. Copper, P = 0.549, time; P = 0.005; copper x time, P = 0.768.

Primiparous cow plasma mineral concentrations were not affected (P > 0.05) by dietary treatment (Table 6.4), with mean values of 17.0, 49.2, 23.0, and 0.28 µmol/L for Cu, Fe, Zn, and Mo respectively. In contrast, there was an effect of time (P < 0.05) on plasma Cu, Fe, Zn and Mo concentrations, where all four were observed to fluctuate throughout the duration of the study, with sharp spikes in both plasma Cu (Week 4; Figure 6.8a) and Zn (Week 2; Figure 6.8b) in cows on either treatment postpartum. Superoxide dismutase, ceruloplasmin activity, and Cp: plasma Cu ratios were not affected (P > 0.05) by treatment with mean values of 2963 U/g of Hb, 20.3 mg/dL, and 1.17 respectively (Table 6.4). There was however an effect (P < 0.001) of time on Cp activity and Cp: plasma Cu ratios (Figure 6.9a,b), both of which increased between weeks -6 and 0, with a subsequent decline thereafter.

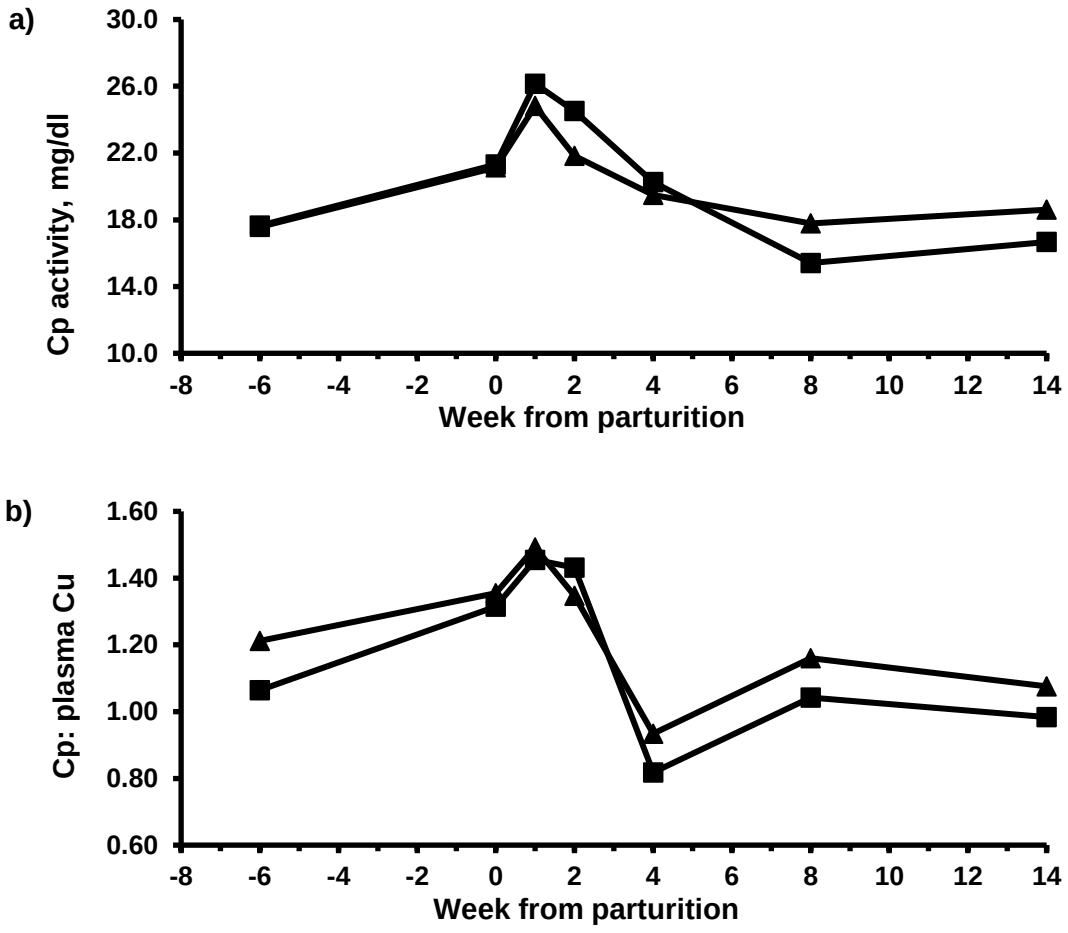

Figure 6.9. Ceruloplasmin activity (Cp activity; a) and ceruloplasmin activity: plasma copper ratio (Cp: plasma Cu; b) of primiparous dairy cows from housing until week 14 of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For Cp activity; pooled SED =2.25. Copper, P = 0.947, time; P < 0.001; copper x time, P = 0.370. For Cp: plasma Cu ratio; pooled SED = 0.113. Copper, P = 0.369, time; P < 0.001; copper x time, P = 0.548.

Table 6.5. Haematological profile of primiparous dairy cows from housing until week 14 of lactation, that were fed either a control or a high dietary copper concentration from 4 to 22 months of age.

	Cu supplementation level			P-values[1]		
Item[2]	C	H	SED	Cu	T	Cu x T
WBC, $10^3/mm^3$	11.6	10.5	0.66	0.261	<0.001	0.320
Mon No, $10^3/mm^3$	0.46	0.42	0.043	0.447	0.027	0.621
Neu No, $10^3/mm^3$	6.43	6.15	0.536	0.609	<0.001	0.164
Lym No, $10^3/mm^3$	4.75	3.80	0.451	0.049	0.037	0.772
Eo No, $10^3/mm^3$	0.07	0.09	0.018	0.467	<0.001	0.066
RBC, $10^6/mm^3$	7.92	7.93	0.226	0.979	0.010	0.276
HCT, %	38.9	40.2	1.12	0.257	0.002	0.262
Hb, g/dL	11.0	11.2	0.28	0.521	<0.001	0.521

[1]Cu= main effect of Cu source supplementation level, T = effect of time, Cu x T = interaction between supplementation level and time.

[2]White blood cells (WBC), monocyte numbers (Mon No), neutrophil numbers (Neu No), lymphocyte numbers (Lym No), eosinophil numbers (Eo No), basophil percentage (Ba), red blood cell count (RBC), haematocrit percentage (HCT), and haemoglobin (Hb).

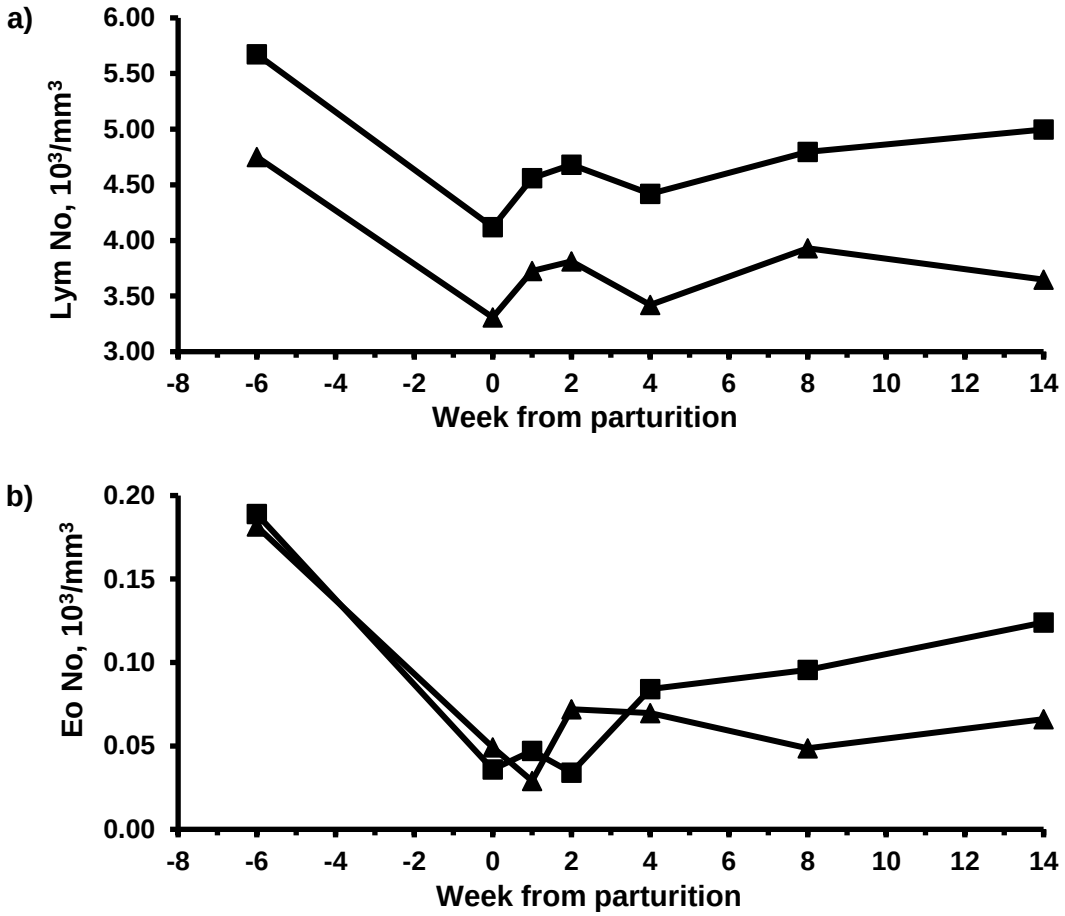

Figure 6.10. Lymphocyte (a; Lym No) and eosinophil (b; Eo No) numbers in primiparous dairy cows from housing until week 14 of lactation, that were fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For Lym no; pooled SED = 1.26. Copper, P = 0.049, time; P = 0.037; copper x time, P = 0.772. For Eo No; pooled = 4.47. Copper, P = 0.467, time; P < 0.001; copper x time, P = 0.066.

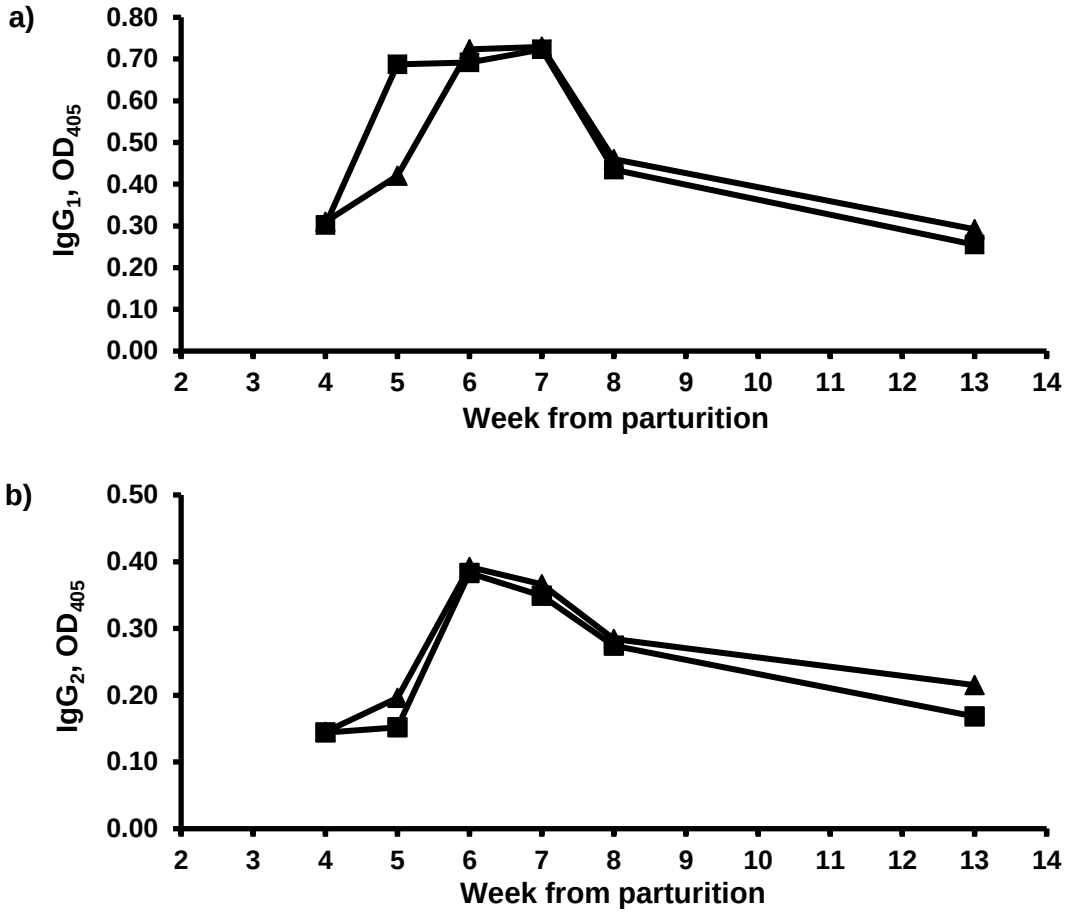

Figure 6.11. Anti-keyhole limpet haemocyanin (KLH) IgG_1 (a) and IgG_2 (b) antibody responses (mean OD_{405}) at week 4 of lactation in primiparous cows fed either a control (▲) or a high (■) dietary copper concentration from 4 months of age until week 14 of lactation. For IgG_1; pooled SED = 0.059. Copper, P = 0.376; time, P < 0.001; copper x time, P = 0.008. For IgG_2; pooled SED = 0.076. Copper, P = 0.679; time, P < 0.001; copper x time, P = 0.886.

6.3.3 Haematological profile and KLH immune challenge

Despite the lack of a treatment effect (P > 0.05) on all other haematological parameters (Table 6.5), there was a trend (P < 0.1) for cows on treatment C to have 4.28 $10^3/mm^3$ lower lymphocyte numbers compared to those on H across the duration of the study (Figure 6.10a). There was also an effect (P < 0.05) of time on all haematological parameters which fluctuated throughout the duration of the study, and a trend (P < 0.1) for a treatment x time interaction for eosinophil numbers, which increased for cows on treatment H at week 8 (0.10 vs. 0.05 for H and C) and 14 (0.12 vs. 0.07 for H and C respectively) of lactation compared to those on C (Figure 6.10b). Cow anti-KLH IgG_1 and IgG_2 responses following immunisation at week 4 of lactation were not affected (P > 0.05) by treatment (Figure 6.11b), although there was an effect of time (P < 0.001) on both parameters, with an increased response between week 0 and 4, and a subsequent sequential decrease thereafter. There

was also a treatment x time interaction ($P < 0.05$) on the IgG_1 response which increased to a greater extent at week 5 of lactation for cows on H compared to those on C.

6.3.4 Hepatic mineral concentrations

There was an effect ($P < 0.001$) of treatment on initial hepatic Cu concentration which was 165 mg/kg DM higher in cows on treatment H compared to C, with mean concentrations of 293 and 128 mg/kg DM for H and C respectively (Table 6.6). Hepatic Cu concentrations then increased ($P > 0.05$) for cows receiving either treatment over the duration of the study to final concentrations of 607 and 424 mg/kg DM for H and C respectively ($P < 0.01$). Finally, there was no effect ($P > 0.05$) of treatment on hepatic Fe, Mo, or Zn concentrations at either sampling point, or their rate of change between sampling points.

Table 6.6. Housing (6-weeks prepartum) and final liver mineral concentrations of primiparous cows fed either a control or a high dietary copper concentration from 4 months of age until week 14 of lactation.

	Cu supplementation level			
Item[1]	C	H	SED	P-value
Initial Cu, mg/kg DM	128	293	27.0	<0.001
Final Cu, mg/kg DM	424	607	45.0	0.004
Δ Cu, mg/kg DM/d	2.30	2.33	0.374	0.936
Initial Fe, mg/kg DM	394	421	39.2	0.513
Final Fe, mg/kg DM	287	293	19.4	0.751
Δ Fe, mg/kg DM/d	-0.83	-0.94	0.197	0.573
Initial Mo, mg/kg DM	3.42	3.30	0.144	0.423
Final Mo, mg/kg DM	4.23	4.02	0.235	0.392
Δ Mo, µg/kg DM/d	6.32	5.38	1.450	0.532
Initial Zn, mg/kg DM	127	115	12.1	0.367
Final Zn, mg/kg DM	224	231	16.5	0.714
Δ Zn, mg/kg DM/d	0.76	0.85	0.144	0.507

[1] Initial = housing, final = week 14.

6.4 Discussion

6.4.1 Performance, energy balance, blood metabolites, and fertility

This study is the first to determine the effect of periparturient and early lactation Cu supplementation on replacement heifer performance and health. To date, studies that have examined the effects of Cu supplementation during lactation have been short term in duration (Engle *et al.*, 2001), neglected aspects relating to health (Chase *et al.*, 2000), and have not examined the potential secondary effects that Cu supplementation earlier in the production cycle may have on lactation (Chapter 5; Engle *et al.*, 2001). The basal TMR (C) had a mean Cu concentration of 14.6 mg/kg DM over the twenty-week duration of the study, which was similar to the target of 15 mg/kg DM predicted to meet cow requirements according to ARC (1980), and NRC (2001; Table 2.4). Based upon estimated DM intakes pre- and postpartum (NRC, 2001; Hoffman *et al.*, 2008), cows on H were predicted to receive an additional 9.7 mg Cu/kg DM over the duration of the study. Using the rate of release of CuO stated by the manufacturer (Agrimin Ltd, Kirmington, UK), predicted DM intake prepartum (Hoffman *et al.*, 2008), and measured DM intake postpartum, cows on treatment H received an additional 8.8 mg Cu/kg DM resulting in a total dietary Cu concentration of 23.3 mg Cu/kg DM. The 8.8 mg Cu/kg DM of additional Cu received by animals on H was therefore similar to the target of 9.7 mg Cu/kg DM.

As previously discussed in Chapter 5, Cu tends to be supplemented as $CuSO_4$ to dairy cows on farm due to its low cost (Brown and Zeringnue, 1994), and high apparent availability (Kegley and Spears, 1994). Copper oxide powder has a lower bioavailability in comparison to $CuSO_4$ (Kegley and Spears, 1994), with the relative bioavailability of CuO powder being reported as 7%, when $CuSO_4$ was used as the reference source (Kegley and Spears, 1994). It could therefore be argued that the CuO used in this study is not comparable to the $CuSO_4$ likely to have been used on farms surveyed by Sinclair and Atkins (2015). The supply however of CuO in this study as an intra-ruminal bolus is thought to negate these differences in bioavailability (Langland *et al.*, 1989). The main issue with suppling CuO as a powder is the fast passage rate through the digestive tract resulting in limited Cu absorption (Langland *et al.*, 1989). In contrast, CuO needles are considered to embed in the gastrointestinal tract resulting in an increase in Cu solubility and apparent absorption (Kegley and Spears, 1994). For example, Yost *et al.* (2002) did not find any difference in hepatic Cu concentration in Holstein-Friesian heifers when a basal diet containing 4.81 mg Cu/kg DM was supplemented with Cu either as an intra-ruminal bolus containing 20 g of CuO needles estimated to provide an additional 13.4 mg Cu/kg DM (Copasure, Animax Ltd; Hoffman *et*

al., 2008), or with 15 mg Cu/kg DM supplied as $CuSO_4$ powder. It would therefore be reasonable to assume that any discrepancies in Cu absorption in the current study due to the use of CuO as opposed to the commercially favoured $CuSO_4$ may have been minimal (Brown and Zeringue, 1994; Kegley and Spears, 1994).

The absorption of Cu in ruminants is not only determined by the quantity and form in which Cu is supplied but the presence of Cu absorption antagonists such as Fe, S and Mo (Suttle, 1991; Suttle, 2010). The dietary concentrations of these antagonists across the duration of this study were typical of that fed on dairy farms in the UK (Sinclair and Atkins, 2015; Sinclair *et al.* 2013). For example, mean Mo and S concentrations were 1.3 mg/kg DM and 2.1 g/kg DM for the twenty-week study period respectively. This concentration of Mo was 0.2 mg/kg DM higher than that reported (1.1 mg/kg DM) on UK dairy farms by Sinclair and Atkins (2015), and the S concentration was typical of that fed in previous studies (1.2 to 2.4 g/kg DM; Sinclair *et al.*, 2013; Sinclair *et al.*, 2017). The mean dietary Fe concentration was 199 mg/kg DM for the twenty-week period, which was 117 mg/kg DM lower than the mean of 316 mg/kg DM fed to dairy cows in the UK during the winter period (Sinclair and Atkins, 2015). This difference in dietary Fe concentrations is unlikely to have impacted dietary Cu absorption, as it is perceived that dietary Fe has little influence below 500 mg Fe/kg DM. In the current study, cows were fed the same basal diet, and therefore animals on either treatment received the same concentration of dietary antagonists (Suttle, 2010). The effects that these dietary antagonists may have had on apparent Cu absorption coefficients between the three studies (Chapter 4, 5, and 6), as well as interactions with dietary starch and sugars have been discussed in Chapter 7.

The current study differs from previous studies examined the effects of Cu supplementation upon lactation in two ways (Chase *et al.*, 2000; Engle *et al.*, 2001). Firstly, cows entered this study with initial hepatic Cu concentrations that differed due to the continuation of the treatments administered in Chapter 5. This meant that differences in performance resulting from hepatic Cu concentration could be evident from the beginning of this study, in comparison to other studies where there was no difference in initial hepatic Cu concentration (Engle *et al*., 2001). Secondly, the effects of Cu supplementation on performance parameters at the end of Chapter 5 such as condition score, were present at the start of this study, and enabled the evaluation of any secondary effects that Cu supplementation during the growing phase may have had upon lactation, as well as interactions between these secondary effects (CS), dietary Cu concentration and/or hepatic

Cu concentration. This type of evaluation would not be possible in a traditional balancing of initial performance and vary dietary Cu concentration type of study (Engle *et al.*, 2001).

Primiparous cows on C had higher yields of milk, protein, and lactose in week 2 of the study, combined with a reduced negative energy balance during lactation than those on H. There are two potential explanations for these effects, one relating to the additional dietary Cu supplied to cows on H (Durand and Kawashima, 1980), and the second relating to the higher CS of animals on H at the beginning of lactation (Lie *et al.*, 2017; Roche *et al.*, 2009). There is little evidence to suggest an effect of dietary Cu concentration on lactation performance in dairy cows (Chase *et al.*, 2000; Engle *et al.*, 2001). For example, Engle *et al.* (2001) reported no effect of Cu supplementation on milk composition or yield of Holstein cows when a basal diet containing 8.9 mg Cu/kg DM was supplemented with either 10 or 40 mg Cu/kg DM as $CuSO_4$ to provide total dietary concentrations of 18.9 and 48.9 mg Cu/kg DM respectively. Additionally, Chase *et al.* (2000) reported no effect of dietary Cu concentration on the lactation performance of Holstein cows when a diet containing 8 mg Cu/kg DM was supplemented with either 15 or 30 mg Cu/kg DM as $CuSO_4$ to provide a total dietary Cu concentrations of 23 and 38 mg/kg DM respectively. In contrast, there is some evidence to suggest a negative effect of dietary Cu concentration on the performance of other breeds of growing cattle (Arthington, 2005; Engle and Spears, 2000). Arthington (2005) reported a decreased apparent digestibility of NDF and CP when Brangus steers were fed a basal diet containing 8.65 mg Cu/kg DM supplemented with 12.5 g of CuO as an intra-ruminal bolus (Copasure, Animax, Ltd), predicted to supply an additional 17.3 mg Cu/kg DM to result in a total dietary Cu concentration of 26.0 mg/kg DM. Durand and Kawashima (1980) extensively reviewed the effects of dietary Cu supply on ruminal fermentation both *in vivo and in vitro*, and concluded that the potential of Cu to either decrease or increase cellulolysis depended on a variety of dietary factors such as the dietary concentration of other minerals. McNaught *et al.* (1950) also identified the potential of excess dietary Cu to reduce ruminal protein digestibility. It is therefore possible that despite the cows used in the current study being Holstein, decreased digestibility due to increased ruminal Cu in cows on H resulted in decreased energy availability and a greater negative energy balance during lactation (Arthington, 2005).

The effects of CS at calving on lactating dairy cows have been well documented (Roche *et al.*, 2009; Stockdale, 2001), and the greater CS of cows on H at parturition in combination with increased hepatic Cu concentrations may be another possible explanation for their decreased performance compared to those on C (Lie *et al.*, 2017; Roche *et al.*, 2009). It is

now generally accepted that there is a curvilinear association between CS and milk production (Stockdale. 2001; Broster and Broster, 1998), where increasing CS increases milk production up to an optimal value of 3.5 (Roche *et al.*, 2007), with a subsequent decline in production at higher condition scores (Waltner *et al.* 1993). Increased CS at parturition allows for increased lipolysis which acts as the primary energy source for non-mammary tissues during early lactation (Bauman and Currie, 1980), and increases the quantity of glucose available for lactose mammary synthesis thereby increasing milk yield (Bauman and Currie, 1980). Dry matter intakes during early lactation are also influenced by CS (Roche *et al.*, 2009), adipose tissue decreases leptin secretion at higher condition scores (Caldeira *et al.*, 2007). Leptin is thought to be the primary regulator of intake (Zhang *et al.*, 1994), and as a consequence, DMI decreases (Caldeira *et al.*, 2007; Zhang *et al.*, 1994). Indeed, it is hypothesised that as CS approach optimal (Roche *et al.*, 2009), the energy gain from increased lipolysis is offset by decreased DMI resulting in decreased milk production (Garnsworthy and Topps, 1982).

Given that cows on H had a higher CS than those on C at calving, and there was no difference in DMI between the treatments at the beginning of lactation, it is therefore surprising that cows on H did not perform as well as those on C during lactation (Roche *et al.*, 2009). This outcome may however be a result of the increased hepatic Cu concentration of cows on H (Lie *et al.*, 2017), although evidence in ruminants is scarce (Suttle, 2010), there are reports that the hepatic enzyme carnitine palmitoyltransferase-1 (CPT-1) can be upregulated at increased hepatic Cu concentrations (Lie *et al.*, 2017). Carnitine palmtoyltransferase-1 (CPT-1) is responsible for the transportation of cytosolic fatty acids into the mitochondria where they undergo β-oxidation (McGarry and Brown, 1997). The complete β-oxidation of fatty acids in the liver is necessary to generate adenosine triphosphate (ATP) via the citric acid cycle and the electron transport chain (Stipanuk, 2000). In periods of negative energy balance, the activity of CPT-1 has been shown to increase with a subsequent increase in β-oxidation rates (Jesse *et al.*, 1986). During this process, hepatic cells convert excess acetyl CoA into the ketone bodies acetoacetate and BHB (Herdt, 2000), which when oxidised act as an energy source for vital organs not capable of fatty acid mobilisation as exemplified the brain (Stipanuk, 2000). The generation of energy via ketogenesis however is much less energy efficient than complete β-oxidation (Stipanuk, 2000). This may explain the decreased energy efficiency of cows on H (Herdt, 2000), where increased hepatic Cu increased the activity of CPT-1 (Lie *et al.*, 2017), resulting in a greater proportion of NEFA being converted into less energy efficient ketone bodies (Drackley, 1999; Stipanuk, 2000). The demand for energy in these animals on H

would subsequently have been greater (Sumner and McNamara, 2007), and subsequently increased CS loss across the lactation section of the study (Roche *et al.*, 2009).

This greater loss in CS results in a situation where hepatic mitochondria become saturated with NEFA that can't be processed by β-oxidation (Drackley, 1999). Excess NEFA can be dealt with by the liver in two ways (Drackley, 1999; Roche *et al.*, 2009). Firstly, it can be oxidised via peroxisomes to produce hydrogen peroxide and heat, a much less efficient pathway in comparison to β-oxidation (Drackley, 1999; Singh, 1997). Secondly, it can be re-esterified and released into the bloodstream as very low-density lipoproteins (VLDL; Roche *et al.*, 2009). Bovines are able to increase re-esterification rates during states of negative energy balance (Bauchart, 1993), it is however thought that cattle are limited in their ability to export VLDL from the liver due to a reduced capacity to synthesise apoprotein B (Bauchart, 1993; Avramoglu and Adeli, 2004), a key component in the process of hepatic VLDL secretion (Avramoglu and Adeli, 2004). Increased NEFA uptake by the liver combined with inefficient VLDL release results in the accumulation of triglyceride within hepatocytes and at its extreme causes fatty liver (Roche *et al.*, 2009). Increased peroxisomal oxidation and/or an accumulation of hepatic triglyceride may subsequently explain the apparent inefficiency of cows on treatment H (Roche *et al.*, 2009).

Increased hepatic synthesis of BHB in animals on H may also explain the reduced milk urea concentrations of cows on H compared to those on C (Stipanuk, 2000; Mew *et al.*, 2017). The hepatic mitochondrial conversion of ammonia to carbamoyl phosphate within the urea cycle requires acetyl CoA (Mew *et al.*, 2017), and so the diversion of acetyl CoA to BHB reduces the quantity of acetyl CoA available for urea synthesis (Herdt, 2000), resulting in decreased blood and therefore milk urea concentrations of cows on H (Butler *et al.*, 1996; Hennessy and Nolan, 1988). Given the increased plasma BHB, CS loss, and decreased milk urea in cows on H, a decrease in the efficiency of fatty acid metabolism seems likely (Butler *et al.*, 1996; Roche *et al.*, 2009).

Plasma BHB concentrations were increased for cows on treatment H (Stipanuk, 2000), and following production in the liver (White, 2015), this metabolite is transported through the body where it acts as an energy source (Stipanuk, 2000). The ability to use plasma BHB and NEFA concentrations as markers of negative energy balance in transition cows (Wankhade *et al.*, 2017), and the potential negative effects that elevated concentrations may have for cow health and fertility have been well documented (Butler, 2005; Ospina,

2010; McArt *et al.*, 2012). Ospina (2010) sampled 2758 transition cows pre- and postpartum, and established critical threshold disease risk values of 0.60 and 0.96 mmol/L postpartum for NEFA and BHB respectively. Cows with concentrations in excess of these values were 4 times more likely to suffer from diseases such as ketosis or displaced abomasum, and 13-16% less likely to conceive (Ospina, 2010). There is however some debate over these risk values, McArt *et al.* (2012) defined sub-clinical ketosis to have occurred when plasma BHB concentrations ranged from 1.2 to 2.9 mmol/L. Despite these discrepancies in defining sub-clinical ketosis (Ospina, 2010; McArt *et al.*, 2012), overall disease risk to cows on either treatment in the current study was likely to be low (Ospina, 2010; Wankhade *et al.*, 2017), as mean NEFA concentrations of 0.68 mmol/L were above the 0.60 mmol/L threshold at parturition but decreased rapidly thereafter to 0.25 mmol/L by week 4 of lactation. In contrast, other studies have reported mean NEFA concentrations above 0.60 mmol/L throughout the first 70 days of lactation (Little *et al.*, 2017). Indeed, at no point were plasma BHB concentrations of cows on either treatment above the critical threshold value of 0.96 mmol/L defined by Ospina, (2010). Differences in health and fertility between cows on the different treatments as a result of differences in energy balance were unlikely (Nydam *et al.*, 2013; Wankhade *et al.*, 2017), and although elevated hepatic Cu concentrations did not increase ketosis risk in this study (Ospina, 2010). It may serve to increase the risk of clinical ketosis in situations where cows are already predisposed to the condition e.g. over-conditioning at parturition (Vanholder *et al.*, 2015).

Fertility measures undertaken in this study should be interpreted within caution due to the potential direct effects of dietary Cu concentration (Hawkins, 2014), and the potential indirect effects of Cu concentration via body reserves, although the differences in body energy reserves between heifers on the two treatments were minor (Wankhade *et al.*, 2017).

6.4.2 Plasma mineral concentrations, Cu-dependent enzymes, and immune challenge

It is generally accepted that plasma Cu is a poor indicator of Cu status (Suttle, 2010; Dias *et al.*, 2013), as concentrations are under homeostatic control by the liver even during instances of Cu repletion or depletion provided total hepatic Cu concentrations are not limiting (Hellman and Gitlin, 2002). There was no effect of treatment on plasma Cu concentration in the current study which were all well above the 9 µmol/L considered to denote the lower limit of adequacy in both cattle and sheep throughout the study (Laven

and Livesey, 2005). These findings support other studies that have reported plasma Cu not to be affected by Cu supplementation (Arthington *et al.*, 2005; Engle *et al.*, 2001). For example, Yost *et al.* (2002) reported no effect of dietary Cu concentration on plasma Cu in Holstein heifers fed a basal ration containing 5.45 mg Cu/kg DM supplemented with 15 or 30 mg of Cu as $CuSO_4$ to give total dietary Cu concentrations of 20.45 and 35.45 mg/kg DM respectively. Dias *et al.* (2013) used a meta-analysis to investigate the relationship between dietary Cu and plasma Cu concentrations in growing cattle, and reported that prediction equations relating the two variables would be of limited use, and it is only during periods of excessively high or low hepatic Cu that plasma Cu concentration may prove useful (Dias *et al.*, 2013). The lack of a response of plasma Cu to changing Cu status in the current study, meant that the relative activities of the Cu-dependent enzymes SOD and Cp were also monitored as potential indicators of Cu status (Suttle, 2010). Perhaps, it is not surprising however that there was no effect of dietary treatment on either enzyme, as Cp activity has been shown to be highly correlated with plasma Cu values (Legleiter and Spears, 2007), and SOD may only respond to a deficiency of Cu over extended time periods (Paynter, 1987).

Normal calving has been shown to induce an acute-phase response through a variety of mechanisms (Jawor and Stefaniak, 2011). The primary cause is thought to be tissue damage caused by the passage of the calf through the cervix and placental expulsion (Uchida *et al.*, 1993), although changes in circulating blood cortisol and prostaglandin concentrations may also exert an influence (Alsemgeest *et al.*, 1993).The ability of Cp to act as a minor acute-phase protein (Kaya *et al.*, 2016), means that it is not surprising that Cp activities for cows on either treatment increased rapidly in the weeks following parturition (Jawor and Stefaniak, 2011; Kaya *et al.*, 2016). No major acute-phase proteins such as haptoglobin were monitored in this study (Plaizier *et al.*, 2009), however increased plasma Cu, and decreased plasma Zn would be expected in combination with increased Cp during an acute phase response (Plaizier *et al.*, 2009; Sattar *et al.*, 1997), both of which were observed for cows on either treatment in the current study.

The generation of the immunoglobulin IgG_1 and IgG_2 isotopes within mammals following immunisation is widely used as an indicator of the Thymus helper cell (Th-cell) balance within the animal (Firacative *et al.*, 2018), where an increased IgG_1 response is considered to denote an upregulation of Th2 (Firacative *et al.*, 2018), and an increased IgG_2 response is thought to signify an upregulation of Th1 (Firacative *et al.*, 2018). Ward and Spears (1999) reported an increased Th2 response in Angus steers following immunisation with ovalbumin

when 5 mg Cu/kg DM was added to a basal diet containing 5.2 mg Cu/kg DM to give a total dietary concentration of 10.2 mg Cu/kg DM. An increased Th2 response, and a decreased Th1 response were observed in Holstein-Friesian heifers following immunisation with ovalbumin in Chapter 5, when a basal diet containing 16.2 mg Cu/kg DM was supplemented with 15.6 mg Cu/kg DM to give a total dietary concentration of 31.8 mg Cu/kg DM. The hypothesis given for the increased Th2 response observed within supplemented heifers in Chapter 5, related to the activation of nuclear factor kappa light chain enhancer of B cells (NF-κB) by increased hepatic Cu concentrations (Cisternas *et al.*, 2005). This activation of NF-κB may subsequently have reduced the secretion of transforming growth factor 1 (Bellone *et al.*, 1995), leading to a decreased Th1 response (Bellone *et al.*, 1995). In addition, Th1 cells have the ability to inhibit Th2 cell formation (Gajewski and Fitch, 1988), and so a decreased Th1 response removes this inhibition allowing for an increased Th2 response (Spellberg and Edwards, 2001). It is surprising therefore that cows on H only had an increased Th2 response at week 5 of lactation in the current study, and there was no effect of treatment on the Th1 response at any time point. Reasons for this lack of response may however reside with the increasing hepatic Cu concentrations of cows on either treatment during the lactation section of this study (Ward and Speers, 1999; Fuentealba *et al.*, 1993), where above a critical hepatic Cu concentration there is no further increase in NF-κB activity (Lilienbaum and Israël, 2003). This plateau may either be a result of Cu storage location within the cell (Fuentealba *et al.*, 1993), with a greater proportion of Cu being stored in the lysosomal fraction at high hepatic Cu concentrations (Cherian and Nordberg, 1983), or be a rate limiting effect of another factor involved in NF-κB activation (Hayden and Ghosh, 2004). Plateaus in NF-κB activity have yet to be reported with respect to Cu (Kudrin, 2000), but there are examples relating to other elements such as calcium (Lilienbaum and Israël, 2003). The increased lymphocyte count observed for cows on H were most likely an historical effect of hepatic Cu on NF-κB activity both in Chapter 5 and at the beginning of this study (Kerl *et al.*, 1986; Tough and Sprent, 1995). The relatively long (months) lifespan of some lymphocyte subsets (Tough and Sprent, 1995), mean that significant changes in circulating lymphocyte numbers by the end of this study as a result of increased hepatic Cu are unlikely (Kerhl *et al.*, 1986).

6.4.3 Hepatic mineral concentration and enzymes relating to liver function

The liver is generally regarded as the primary organ responsible for both Cu homeostasis and storage within cattle (Laven and Livesey, 2005), and when dietary Cu is supplied in excess of the animal's requirements, one of the most significant biochemical changes is an increasing hepatic Cu concentration (Suttle, 2010). The increased hepatic Cu

concentrations in cows on H as a result of supplementing Cu as an intra-ruminal CuO bolus is not surprising and has already been discussed (Kegley and Spears, 1994; Yost *et al.*, 2002). It is surprising however that the hepatic Cu concentration of cows on either treatment increased to such an extent between housing (128 and 293 mg Cu/kg DM for C and H respectively; Chapter 5) and the end of this study (424 and 608 mg Cu/kg DM for C and H respectively), given that they remained relatively constant between 13 months of age (195 and 350 mg Cu/kg DM for C and H respectively) and 6-weeks pre-calving in chapter 5, on similar dietary Cu concentrations. The increase to 424 and 608 mg Cu/kg DM for C and H respectively by the end of this study meant that hepatic Cu concentrations of cows on H were above the 508 mg/kg DM threshold thought to put animals at risk of developing clinical Cu toxicity (Livesey *et al.*, 2002). This accumulation of hepatic Cu in cows on either treatment (Johnston *et al.*, 2014), may have caused hepatocytes to rupture, releasing liver enzymes into the bloodstream, and resulting in elevated serum GLDH concentrations (Giannini *et al.*, 2005). Reasons for this increase in hepatic Cu concentration of cows on either treatment during lactation most likely relates to the high starch content of the lactating TMR in this study compared to the diets fed in Chapter 5 (Gould and Kendall, 2011; Drewnoski *et al.*, 2014), although cross-study comparisons in diet composition are discussed at length in Chapter 7. It is however surprising that the difference in hepatic Cu concentrations between the two treatments remained relatively stable throughout both Chapter 5 and this study, with differences in hepatic Cu concentrations at 7, 13 months of age, housing, and week 14 of lactation of 155, 155, 165, and 183 mg/kg DM respectively. Animals on treatment H received dietary Cu concentrations that were approximately 2.5 their predicted requirement during the growing phase (Chapter 5), and 1.6 times their predicted requirement in the current study respectively (ARC, 1980; NRC 2001). It would therefore be expected that hepatic Cu concentrations between animals on the different treatments would continue to diverge throughout the growing and lactating studies rather than plateau (Suttle, 2010; Engle *et al.*, 2001). This is not however the first study to report a plateau in hepatic Cu concentrations when dietary Cu is supplied in excess of the animal's requirements (Arthington, 2005; Yost *et al.*, 2002). Yost *et al.* (2002) reported an increase in hepatic Cu of Holstein heifers fed a basal ration containing 4.81 mg Cu supplemented with 25 g of CuO as an intra-ruminal bolus until day 28 of the study, with a plateau in concentrations thereafter. The biological mechanisms behind this apparent adjustment of the liver to dietary Cu concentration remain unclear (Yost *et al.*, 2002), however it is hypothesised that there is an excretory and/or absorption mechanism by which the body regulates apparent Cu absorption in order to prevent clinical Cu toxicity (Stoszek *et al.*, 1986). For instance, it is thought that ATP7B may be able to translocate within the

hepatocyte and increase biliary Cu excretion (Hernandez *et al.*, 2008), however evidence for this type of phenomena in ruminants is scarce (Suttle, 2010).

6.5 Conclusions

Feeding Holstein-Friesian primiparous cows dietary Cu concentrations above predicted requirement throughout the rearing phase and first 14 weeks of lactation decreased early lactation performance such as lactose, milk, and protein yields. It also increased the negative energy balance that cows experienced during early lactation resulting in a greater mobilisation of body tissue to maintain performance. The predictive measure of transition cow health, plasma BHB was within clinical thresholds, but higher in cows fed increased dietary Cu concentrations. Reasons for these differences in energy balance due to dietary Cu concentration are not clear and require further investigation. The immune system of these first lactation cows was not modulated by increasing dietary Cu concentration.

CHAPTER 7: General Discussion and conclusions

7.1 General discussion

This thesis has characterised the effect of rumen pH and/or dietary starch concentration to influence Cu status during lactation (Chapter 4), and the effects of supplementing Cu in excess of requirement on performance, health and fertility throughout the rearing phase (Chapter 5), and the first 14 weeks of lactation (Chapter 6). These chapters combine to provide contrasting evidence on widely held views with respect to Cu absorption in ruminants (Suttle, 1991; Suttle, 2010). Studies with monogastric laboratory animals have shown that Cu absorption is facilitated by both specific (copper transporter 1; Ctr 1) and non-specific transport (divalent metal transporter; DMT) proteins within the mucosa of the intestines, and the former is hypothesised to vary depending upon animal requirements (Prohaska, 2006). In weaned ruminants however, it is thought that Cu availability is determined primarily by digestive processes in the rumen, where Cu can interact with antagonists such as S, Mo, and Fe (Suttle, 1991; Gould and Kendall, 2011). These interactions have since become the primary focus of reviews and textbooks relating to the subject area (NRC, 2001; Suttle, 2010). Indeed, Suttle (1991) went as far as to state that in ruminants, there is no other interaction, with the capability of Cu-S-Mo to sway the nutritional status of the animal from toxicity to deficiency, when a natural foodstuff is consumed. The results of this thesis and indeed recent publications would suggest that there are more variables than just S and Mo involved in this hypothesis (Suttle, 2016; Sinclair *et al.*, 2017).

Evidence that other dietary factors may have an equivalent role in Cu absorption originated as early as the 1980s, when Suttle (1983) reported a decreased effect of S and Mo on Cu availability in hay compared to fresh grass when fed to sheep. More recent studies have provided further evidence, with Sinclair *et al.* (2017) reporting a decrease in the hepatic Cu concentration in lactating dairy cows when a grass-silage- compared to a maize silage-based diet was fed with additional S and Mo. In trying to determine reasons for these differences in Cu availability between grass and maize silage-based rations, increasing dietary starch and/or reducing rumen pH was demonstrated to increase hepatic Cu retention in Chapter 4. The consequences that rumen pH and/or dietary starch concentration may have on hepatic Cu concentrations throughout the production cycle were then demonstrated in the transition to higher starch between Chapters 5 and 6. The mean dietary concentrations of S and Mo fed to growing heifers in Chapter 5 (2.29 g S/kg DM and 1.6 mg Mo/kg DM; predicted apparent absorption coefficient of 0.0422), where slightly higher than those fed during the lactating experiment in chapter 6 (2.07 g S/kg DM and 1.3 mg

Mo/kg DM; predicted apparent absorption coefficient of 0.0452; Suttle and McLauchlin, 1976), however this was offset by a higher mean dietary Cu concentration in Chapter 5 (16.2 vs. 14.5 mg Cu/kg DM in Chapters 5 and 6 respectively). This meant that available Cu during the growing and lactating phases as a result of S and Mo antagonism was almost identical at 0.68 mg Cu/kg DM in chapter 5, and 0.66 mg Cu/kg DM in Chapter 6 (Suttle and McLauchlin, 1976). Dietary Fe concentrations were however 86 mg/kg DM higher in Chapter 5 compared to 6, there are no equations which predict Cu availability as a result of dietary Fe concentration (Suttle, 2010). It is however unlikely that differences in dietary Fe of this magnitude affected Cu availability in these studies, as Chase *et al.* (2000) reported no difference in hepatic Cu retention when lactating Holstein cows were fed diets supplemented with and without 500 mg Fe/kg DM. Indeed, it does seem that commencing the lactation diet in Chapter 6 was the causal factor for such large increases in hepatic Cu concentrations of heifers on both treatments H and C, with GLDH concentrations increasing rapidly following parturition (Johnston *et al.*, 2014). Given that Cu availability as a result of dietary antagonists was almost identical pre- and postpartum (Suttle, 1991; Suttle and McLauchlin, 1976), other dietary factors may account for this increase in hepatic Cu concentrations by the end of Chapter 6 (Week 14 of lactation). The starch+sugar content of the lactation diet fed in Chapter 6 (228 g/kg DM), was much higher than both the dry cow diet fed in chapter 6 (86 g/kg DM), and the basal ration fed to the growing heifers throughout Chapter 5 (138 g/kg DM). Given that these differences in starch+sugar content are greater than the differences between diets reported in Chapter 4 (170 vs. 242 g/kg DM for LS and HS respectively), it is not surprising that hepatic Cu concentrations of primiparous on either treatment increased during lactation in Chapter 6 (Drewnoski *et al.*, 2014; Clarke and Laurie, 1980).

One factor that may have affected the Cu status of heifers on either treatment in Chapter 5, is the potential of pasture to alter rumen pH and subsequently Cu absorption when heifers were grazed during the summer months (Drewnoski *et al.*, 2014a; Gould and Kendall, 2011; Packer *et al.*, 2011). Packer *et al.* (2011) reported a mean rumen fluid pH of 7.13 when commercial beef Angus and Murray grey steers were grazed on lush pasture composed of Bimbil oats for 56 days, followed by tetraploid annual ryegrass for 35 days, with mean NDF and WSC contents of 399 and 273 g/kg DM respectively throughout the duration of the study. The NDF content of the perennial ryegrass in Chapter 5 is however much higher at 502 g/kg DM, and the WSC content much lower at 161 g/kg DM compared to that reported by Packer *et al.* (2011). Tafaj *et al.* (2007) reported a strong positive correlation between dietary NDF content and rumen pH, and so it is highly unlikely that heifers in Chapter 5 experienced rumen pH values that were as low as the mean pH of 6.39 observed in cows

fed the high starch diets in Chapter 4 (Packer *et al.*, 2011; Tafaj *et al.*, 2007), and so it is also unlikely that decreased rumen pH values at grazing altered hepatic Cu retention (Suttle, 1991; Clarke and Laurie, 1980; Drewnoski *et al.*, 2014a).

This pattern of toxic hepatic Cu concentrations postpartum in Chapter 4 and 6 is reflected in bovine liver designated for human consumption in the UK (Kendall *et al.*, 2015), where following the collection of samples from an abattoir it was reported that approximately 40% of UK Holstein-Friesian cull cows had hepatic Cu concentrations in excess of the 508 mg/kg DM threshold generally accepted to pose a risk of Cu toxicity (Kendall *et al.*, 2015; Livesey *et al.*, 2002). The main accusations directed at the industry for this overloading of Cu relate to the high supplementation levels of dietary Cu at farm level (Sinclair and Atkins, 2015; Kendall *et al.*, 2015). Kendall *et al.* (2015) advocated that the “more is better" approach, was one of the primary reasons for high liver Cu concentrations on farm. Additionally, Sinclair and Atkins (2015) reported a mean early lactation Cu supplementation level of 28 mg/kg DM during the winter on 50 UK dairy farms in central and northern England, which was well in excess of the maximum nutritional guideline of 11 mg Cu/kg DM for these cattle (NRC, 2001; Sinclair and Atkins, 2015).

Evidence from this thesis does however question the importance of a supplementation concentration in relation to Cu toxicity within the industry. For example, heifers on H in Chapter 5 received a dietary concentration of 31.8 mg Cu/kg DM, which was 3.7 times higher than the lowest recommended nutritional guideline of 8.6 mg Cu/kg DM (CSIRO, 2007). Despite this overfeeding, these animals had liver Cu concentrations from 13 months of age until calving that were well below the 508 mg Cu/kg DM suspected to pose a risk of clinical Cu toxicity (Livesey *et al.*, 2002). In contrast, postpartum in Chapter 6, despite cows on C receiving dietary Cu concentrations (14.5 mg Cu/kg DM) that were approximately equivalent to their predicted requirement of 15.7 mg Cu/kg DM (NRC, 2001), mean hepatic Cu concentrations increased to 424 mg Cu/kg DM by week 14 of lactation. The studies in this thesis tend to support Suttle (2016), where the author attributed the upsurge in copper toxicity in cattle to an industry “ignorance” regarding Cu absorption from mixed rations. It is not necessarily the mixed rations per se that are causing hepatic Cu loading, but may be the rumen pH effects that come with them (Clarke and Laurie; Drewnoski *et al.*, 2014a). It therefore stands to reason that other practices that have the potential to influence rumen pH such as cow sorting activity may radically change dietary Cu concentration (Humer *et al.*, 2018). It may be more prudent to consider that although Cu supplementation plays a role in bovine Cu toxicity (Kendall *et al.*, 2015), other dietary factors which have been

modified to meet the needs of the modern high producing dairy cow may be of considerable importance (Humer *et al.*, 2018).

There may also be an issue with the provision of Cu to young calves (Suttle, 2016). Similar to the toxic hepatic Cu concentrations of heifers on either treatment at 7 months of age in Chapter 5, Hunter *et al.* (2013) reported high winter mortality rates in jersey calves receiving a milk replacer which contained 10.5 mg Cu/kg DM, and a creep feed with a Cu content of 70.5 mg Cu/kg DM. The effects of these high dietary concentrations are exacerbated by the relatively high Cu availability within the newborn calf (50-60%) compared to their adult counterparts (1-5%) prior to the development of a fully functioning rumen (Bremner and Dalgarno, 1973). There are no nutritional guidelines regarding the Cu requirements of newborn calves (ARC, 1980; NRC, 2001), but in comparative terms, cow's milk has a Cu content of 0.15 to 0.20 mg Cu/kg (Schwarz and Kirchgessner, 1978), milk replacer when fed at 14% solids would supply approximately 1.5 mg Cu/kg (Dennis *et al.*, 2019), a factor of 10 greater than that supplied by cows' milk (Schwarz and Kirchgessner, 1978). Reducing the Cu loading of artificially reared calves may be particularly challenging for the industry (Hunter *et al.*, 2013 Khan *et al.*, 2016), with several dietary factors that can influence rumen development up to 4 months of age (Khan *et al.*, 2016), combined with creep feeds which may naturally contain high concentrations of Cu (Hunter *et al*, 2013). For instance, Hunter *et al.* (2013) reported that removing Cu supplements from the pelleted creep still resulted in a background dietary Cu concentration of 35 mg/kg DM.

The difficulties associated with the accurate diagnosis of a ruminant's Cu status have been well documented (Suttle, 2010; Williams, 2004), and reviewed in Section 2.8. To date, opinions are divided regarding the accuracy of liver function enzymes to accurately diagnose sub-clinical Cu toxicity (Bidewell *et al.*, 2012; Strickland *et al.*, 2019). Relationships between plasma Cu and serum GLDH in Chapters 4 to 6 have been analysed and presented in comparison to other commonly used indicators of Cu status (Suttle, 2010). The data from Chapters 5 and 6 was combined for analysis (Figure 7.1; Table 7.1), whilst Chapter 4 has been analysed in isolation (Figure 7.2; Table 7.2), under the assumption that heifers in Chapters 5 and 6 were subject to two short-term periods of hepatic Cu loading during early life and the beginning of lactation. It was assumed that cows in Chapter 4 had experienced chronic hepatic Cu loading over a number of years (Sinclair *et al.*, 2013; 2017).

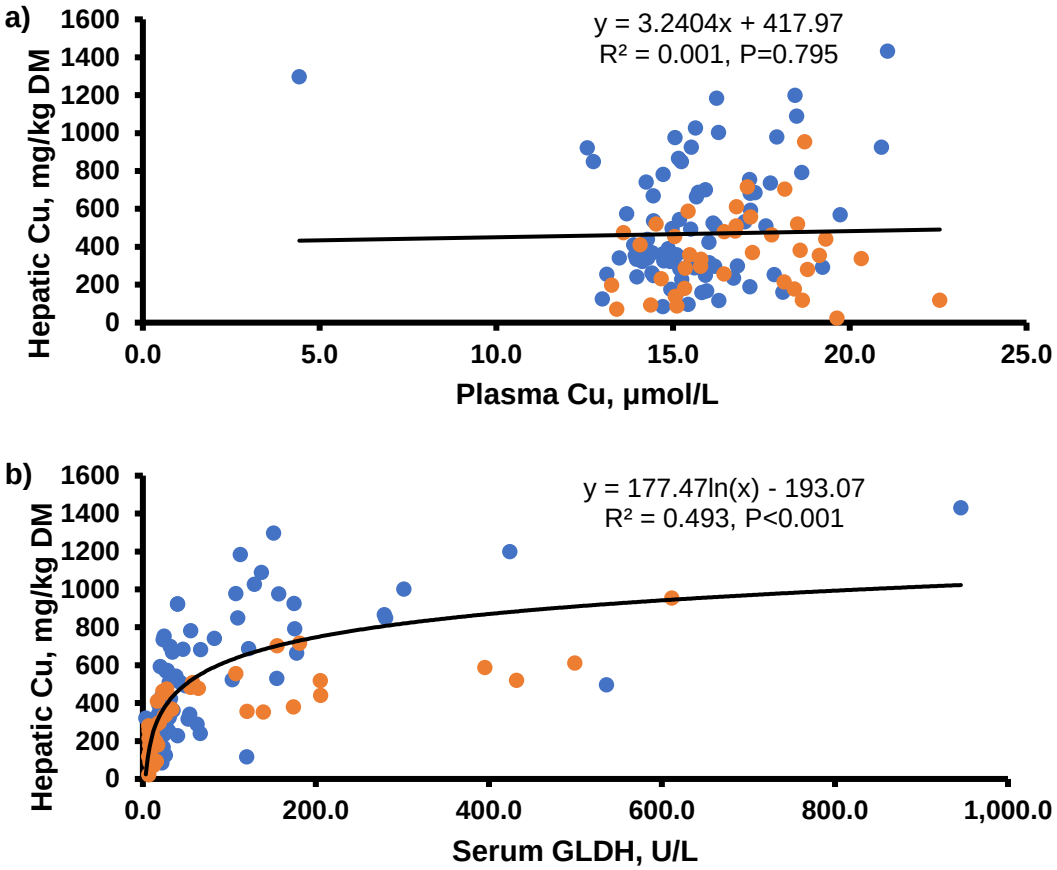

Figure 7.1. Relationship between hepatic copper concentration and plasma copper (Cu; a) or serum glutamate dehydrogenase (GLDH; b) of growing and lactating heifers, at 7 and 13 months of age, 6-weeks prepartum, and week 14 of lactation. For Chapter 5 (●), n = 77. For Chapter 6 (●), n = 38.

Table 7.1. Differential distribution of hepatic and blood indicators of the Cu status of growing and lactating heifers, at 7 and 13 months of age, 6-weeks prepartum, and week 14 of lactation (Chapters 5 and 6).

	% of samples (n = 115)		
Indicator[1]	Deficient	Normal	High
Hepatic Cu mg/kg DM	< 19	19 to 508	> 508
	0 (0)	62 (71)	38 (44)
Plasma Cu, µmol/L	< 9	9 to 19	> 19
	1 (1)	91 (105)	8 (9)
Cp activity, mg/dL	< 12	12 to 24	> 24
	12 (14)	77 (89)	10 (12)
SOD activity[2], U/g of Hb	< 2000	≥ 2000	N/A
	0 (0)	100 (115)	
Serum GLDH[2], U/L	N/A	1 to 25	> 50
		63 (73)	37 (42)

[1] Reference values: hepatic Cu (Laven and Livesey, 2005; Livesey *et al.*, 2002), plasma Cu (Laven and Livesey, 2004; 2005), Cp and SOD activity (Williams, 2004; Telfer *et al.*, 2004), GLDH (Johnston *et al.*, 2014).
[2] N/A = not applicable.

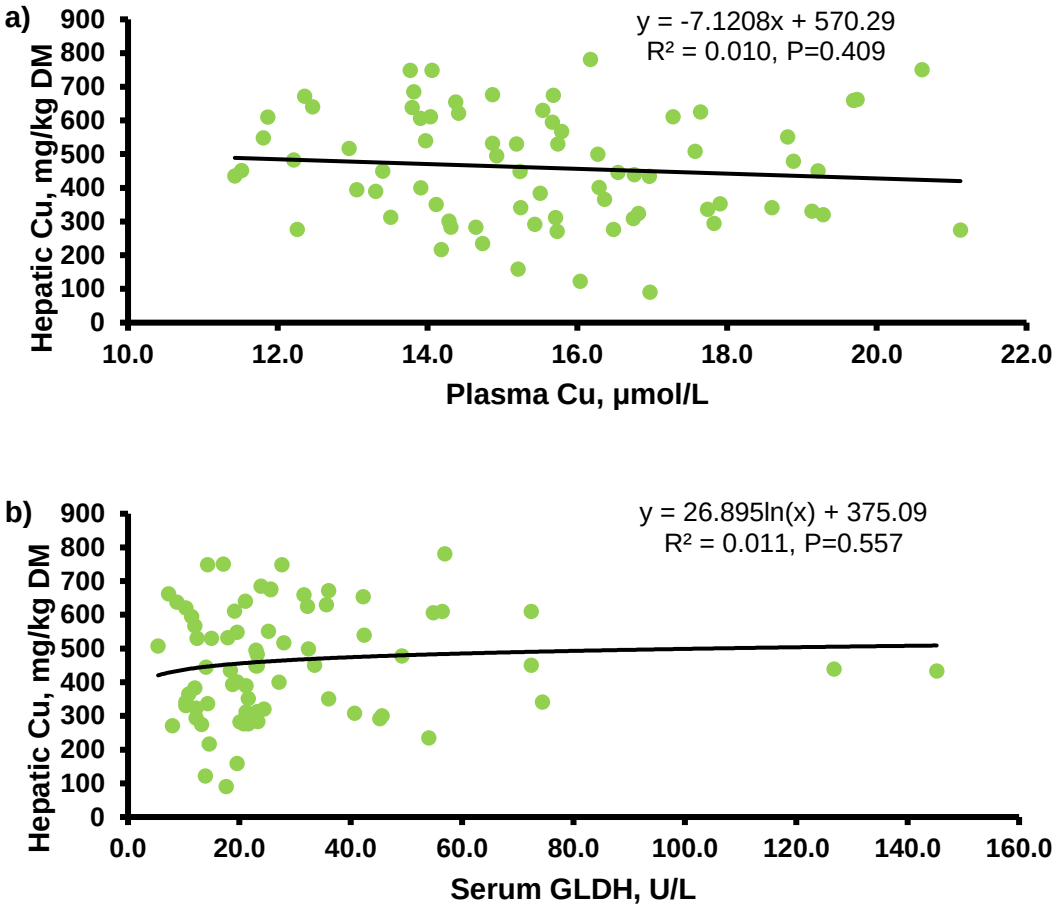

Figure 7.2. Relationship between hepatic copper concentration and either plasma copper (Cu; a) or serum glutamate dehydrogenase (GLDH; b) of multiparous lactating dairy cows. For Chapter 4 (●), n = 73.

Table 7.2. Differential distribution of hepatic and blood indicators of the Cu status of multiparous lactating dairy cows (Chapter 4).

	% of samples (n = 73)		
Indicator[1]	Deficient	Normal	High
Hepatic Cu mg/kg DM	< 19	19 to 508	> 508
	0 (0)	60 (44)	40 (29)
Plasma Cu, µmol/L	< 9	9 to 19	> 19
	0 (0)	90 (66)	10 (7)
Cp activity, mg/dL	< 12	12 to 24	> 24
	4 (3)	73 (53)	23 (17)
SOD activity[2], U/g of Hb	< 2000	≥ 2000	N/A
	29 (21)	71 (52)	
Serum GLDH[2], U/L	N/A	1 to 25	> 50
		88 (64)	12 (9)

[1] Reference values: hepatic Cu (Laven and Livesey, 2005; Livesey *et al.*, 2002), plasma Cu (Laven and Livesey, 2004; 2005), Cp and SOD activity (Williams, 2004; Telfer *et al.*, 2004), GLDH (Johnston *et al.*, 2014).
[2] N/A = not applicable.

Evidence from Chapters 4 to 6 has done little bar reinforce prevailing beliefs relating common indicators of Cu status (Plasma Cu, Cp activity, SOD activity, and hepatic Cu) and clinical pathology (Suttle, 2010; Williams, 2004). The liver is the primary Cu storage organ within the animal (Laven and Livesey, 2005), and as such, hepatic Cu concentration is widely considered to be the most accurate indicator of Cu status (Suttle, 2010). In contrast, plasma Cu is one of the most commonly used diagnostic indicators of Cu status due to its lack of invasiveness (Williams, 2004). Dias *et al.* (2013) related dietary concentrations of Cu, S, and Mo to plasma Cu concentration in a meta-analysis, and concluded that it was only in cases of high or low hepatic Cu concentration that plasma Cu may be used as an accurate indicator of Cu status. Results from the current thesis support this, with no relationship ($P > 0.05$) between plasma Cu and hepatic Cu concentration in either Chapters 5 and 6 ($R^2 = 0.001$; Figure 7.1), or Chapter 4 ($R^2 = 0.010$; Figure 7.2). Plasma Cu concentrations decrease in cases of Cu deficiency as hepatic Cu stores are insufficient to sustain the pool of circulating Cu (Humphries *et al.*, 1983). In contrast, plasma Cu concentrations increase during cases of clinical Cu toxicity where the necrosis of hepatocytes releases Cu into the bloodstream (Suttle, 2010). Given that mean hepatic Cu concentrations of animals in all three experimental Chapters in this thesis were well in excess of the 19 mg Cu/kg DM considered to pose a risk of deficiency, and there were no confirmed cases of clinical Cu toxicity, it is perhaps not surprising that there was no relationship between plasma and hepatic Cu concentrations. Despite 40% of liver samples in Chapter 4 being above the 408 mg Cu/kg DM threshold considered to pose a risk of clinical Cu toxicity (Livesey *et al.*, 2002), only 10% of plasma samples were above the corresponding threshold of 19 µmol/L (Laven and Livesey, 2004).

In terms of the identification of hepatic Cu status, serum Cp activity is thought to offer little advantage over plasma Cu with both parameters being highly correlated (Pearson R = 0.73 to 0.87; Legleiter and Spears, 2007). This correlation is not surprising given that 80-95% of ruminant plasma Cu is present as ceruloplasmin (Suttle, 2010; Terada *et al.*, 1995). There is little evidence from Chapters 5 and 6 that serum Cp activity may be a useful as an indicator of hepatic Cu status. There was also the potential for misleading changes in Cp activity, where decreased Cp activity in Chapter 5 may have been a result of heifer age (Chang *et al.*, 1975), and increased Cp activity in Chapter 6 may have resulted from an acute phase response following parturition (Jawor and Stefaniak, 2011; Kaya *et al.*, 2016). Serum Cp activity has however been advocated as an indicator of high dietary S and Mo concentrations (Mackenzie *et al.*, 1997b), where thiomolybdates formed in the rumen are absorbed into the bloodstream and decrease Cp activity (Telfer *et al.*, 2004). This indicator did indeed prove useful in identifying potential differences in S and Mo metabolism between

the low and high starch diets in Chapter 4. Perhaps the greatest problem with the use of Cp activity as an indicator of Cu status is the range of enzyme assay kits used globally (Laven *et al.*, 2011), and the lack of commercially available standardisation controls making a comparison of values between labs difficult (Laven *et al.*, 2011). Serum Cp activity may therefore be useful as an indicator of differences in thiomolybdate metabolism between treatments within a study (Mackenzie *et al.* 1997b), but of little use between studies (Laven *et al.*, 2011).

Whole blood SOD activity has been advocated as a long-term indicator of Cu deficiency due to the relatively long lifespan of erythrocytes (92 days) in comparison to circulating ceruloplasmin (Suttle and McMurray, 1983; Ward and Spears, 1997). This indicator operates on the principle that dietary Cu intake and hepatic Cu reserves are insufficient to maintain the synthesis of SOD during erythropoiesis (Suttle and McMurray, 1983), and as such the homeostatic control of this mechanism means that there is not a link between SOD activity and Cu toxicity (Suttle and McMurray, 1983). It is not surprising therefore that there were no differences in SOD activity between treatments across the three Chapters in this thesis given that hepatic Cu concentrations were well in excess of the deficiency threshold of 19 mg Cu/kg DM (Laven and Livesey, 2005; Ward and Spears, 1997).

Liver function enzymes including GLDH have been considered more recently as possible indicators of Cu overloading (Bidewell *et al.*, 2012), where liver degeneration during the onset of hepatic necrosis releases these enzymes into the bloodstream (Giannini *et al.*, 2005). The literature however, is conflicting regarding the use of these enzymes (Johnston *et al.*, 2014; Strickland *et al.*, 2019), with some studies identifying them a useful indicators of herd Cu status during cases of suspected over-supplementation (Bidewell *et al.*, 2012; Johnston *et al.*, 2014), whilst others have reported no association between these enzymes and high hepatic Cu concentrations (Strickland *et al.*, 2019). The findings in Chapters 4 to 6 seem equally conflicted. despite the lack of a relationship ($P > 0.05$; $R^2 = 0.011$) between serum GLDH concentrations and hepatic Cu in Chapter 4 (Figure 7.2), there was a relationship ($P < 0.05$; $R^2 = 0.493$) in Chapters 5 and 6 (Figure 7.1). This is also reflected in the differential distribution of the indicators, with 40% of liver samples in Chapter 4 being above the upper threshold of 508 mg Cu/kg DM (Livesey *et al.*, 2002), but only 12% of serum GLDH values were elevated (Johnston *et al.*, 2014). In contrast 38% of liver samples in Chapter 5 and 6 were above the upper threshold, and 37% of GLDH values were elevated. It has been hypothesised previously that discrepancies in the accuracy of this indicator could be a result of differences in mean hepatic Cu concentrations between

studies (Bidewell *et al.*, 2012; Strickland *et al.*, 2019). Evidence from this thesis would suggest that this is not the case, with mean final hepatic Cu concentrations in Chapter 4 being 483 mg Cu/kg DM, compared to 422 mg Cu/kg DM for cows on treatment C in Chapter 6. Elevated serum GLDH concentrations were also noted for cows on C in Chapter 6 but not for cows in Chapter 4 (Bidewell *et al.*, 2012). Similar findings have been noted in other diseases of the liver (Johnston, 1999), where the acute form of the disease provides a greater increase in blood enzyme concentrations relative to the chronic form of the disease (Johnston, 1999). This has also been observed in cases of Cu loading in the liver, albeit not within the ruminant population (Suttle, 2010), where a patient with chronic Wilson's disease was misdiagnosed due to a panel of liver function enzymes that were much lower than expected (Cho *et al.*, 2011). Reasons for this variation in accuracy of liver function enzymes revolves may relate to the type of cell death in each instance (Johnston, 1999). During acute injury of the liver healthy cell death predominantly occurs via necrosis releasing the contents of the cell into the bloodstream (Johnston, 1999). In contrast, during chronic injury, cell death and turnover tends to occur via apoptosis or programmed cell death (Johnston, 1999), part of which includes shutting down the metabolic machinery within the cell, so that when hepatocytes rupture the proportions of these enzymes available to be released into the bloodstream are considerably lower (Haber *et al.*, 1995; Healey *et al.*, 1995). The specificity as well as the accuracy of these enzymes may also be problematic in the diagnosis of sub-clinical Cu toxicity (Du *et al.*, 2017; Johnston *et al.*, 2014). Elevated panels of liver function enzymes have been reported in cattle suffering from ketosis or fatty liver (Du *et al.*, 2017; Sevinc *et al.*, 2001), and in sheep infected with liver fluke (Hodzic *et al.*, 2013). Results from these tests should therefore be interpreted with caution but may prove a useful diagnostic tool when the previous Cu and health status of a herd is known (Hunter *et al.*, 2013; Sevinc *et al.*, 2001).

The recurring theme throughout the chapters in this thesis is that hepatic Cu concentration is a function of the diet supplied, and that changes in performance are likely a function of hepatic Cu concentration (Chapters 5 and 6), provided that hepatic Cu supply is sufficient to prevent Cu toxicity (Suttle, 2010). This may question the experimental design of studies that feed a specific dietary Cu concentration and conclude that it is the concentration in isolation that has affected performance (Chase *et al.*, 2000; Engle *et al.*, 2001). The balancing of Cu status at the beginning of these studies as exemplified by Chase *et al.* (2000), means that hepatic Cu reserves change until they reach a plateau according to the dietary conditions under which they are fed (Yost *et al.*, 2002). In situations such as this, systemic effects of hepatic Cu concentration on performance are difficult to detect as the change in hepatic Cu with time is unknown (Engle *et al.*, 2001). It is surprising that this "hit

and hope" approach is still utilised when studying Cu metabolism, but other sectors of dairy research have moved on (Engle, 2014; Roche *et al.*, 2009). The effect of body condition on lactation performance is a primary example, where a normal experimental practice is to vary condition score to the desired value prior to commencing the study (Roche *et al.*, 2009). There may be justification for using dietary changes in association with liver Cu values to ensure a plateau in hepatic Cu concentration prior to the assessment of performance. This approach used across a range of hepatic Cu concentrations from 19 to 508 mg Cu/kg DM may prove more useful in assessing optimal hepatic Cu concentrations with respect to performance, health and fertility (Laven and Livesey, 2005; Livesey *et al.*, 2002).

7.2 General conclusions

Feeding lactating Holstein-Friesian dairy cows a diet containing 15 mg Cu/kg DM in the absence of significant quantities of S and Mo will result in the hepatic accumulation of Cu, demonstrating that this level of feeding is well in excess of the animal's requirement. In contrast, feeding the same concentration of dietary Cu in a diet with high S and Mo concentrations will cause a decrease in the hepatic Cu of cows fed low but not high starch diets. This difference in apparent Cu availability may be due to the rumen pH modulatory effects of these respective diets. The reasons for these differences in Cu metabolism as a result of dietary starch and/or rumen pH are unclear and require further investigation, but there is a need to take dietary starch concentration and/or rumen pH into account when deciding on appropriate Cu supplementation levels for lactating dairy cows.

Increasing dietary Cu concentrations above the requirement of growing Holstein-Friesian heifers marginally increased performance in terms of growth rate, body condition, and an earlier onset of puberty. In contrast, increasing dietary Cu concentration decreased heifer conception rates. The reasons for these differences in performance as a result of dietary Cu concentration are not clear and require further investigation but may relate to the hepatic synthesis of insulin-like growth factor 1. Additionally, the immune response of heifers was shown to be modulated by dietary Cu concentration, although the most important components of the immune system that are related to long-term health and performance have yet to be validated.

Feeding Holstein-Friesian heifers dietary Cu concentrations above predicted requirement between 4 months of age and week 14 of lactation decreased early lactation performance

such as milk, protein and lactose yields, and increased negative energy balance with a greater mobilisation of body tissue between parturition and week 14. Plasma β-hydroxybutyrate concentration, a predictive measure of transition cow health, was higher in cows fed increased dietary Cu concentrations but were within clinical thresholds. Reasons for these differences in tissue metabolism as a result of dietary Cu concentration are not clear and require further investigation. Increasing dietary Cu concentration during the rearing phase and early lactation failed to modulate the Th1/Th2 balance during peak lactation following immune stimulation.

7.3 Future prospects

It is clear from the Chapters in this thesis that dietary factors other than Mo and S have the potential to influence Cu absorption and metabolism, and there is subsequently a need to determine the effects of diet and stage of production on factors such as rumen pH so that these can be included when deciding appropriate dietary Cu concentrations. This thesis has identified that hepatic Cu concentration may influence the hypothalamic-adrenal-pituitary-axis; the mechanisms of this influence are unclear but may relate to IGF-1 and/or GH, and so there is a need to examine the effects of hepatic Cu concentration on these hormones. It has also identified the potential of hepatic Cu concentration to decrease fertility and to modulate the Th-cell balance when responding to disease. These effects may be secondary, acting via the hypothalamic-pituitary-adrenal axis, or they may primary, with a direct change in reproductive hormones. There is however a need to investigate the effects of hepatic Cu concentration on both reproductive hormones, and others which modulate the immune response. It is clear that increasing hepatic Cu concentration within what is generally accepted as the normal range has the potential to effect measures of fertility and health, and there is therefore a need to investigate further how hepatic Cu concentration can be optimised with respect to health and fertility.

CHAPTER 8: References

Abdel-Misih, S.R.Z. and Bloomston, M.D. 2014. Liver anatomy. *Surgical Clinics of North America,* 90, pp. 643-653.

Adashi, E.Y., Resnick, C.E., D'ercole, A.J., Svoboda, M.E. and Wyk, J.V. Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. *Endocrine Reviews,* 6, pp. 400-420.

Advisory Committee on Animal Feed (ACAF). 2010. Presentation on copper supplementation in animal feed. In: *Minutes of the ACAF Meeting held on 15th Dec 2010,* Aviation House, London: ACAF. Available online: http://acaf.food.gov.uk/acafmeets/acaf_2010_meetings/ acafmeet151210/acafmins1210 [Accessed 22nd October 2016].

Agricultural and Food Research Council (AFRC) 1993. *Energy and protein requirements of ruminants. An advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients.* CAB International, Wallingford, UK.

Agricultural Research Council (ARC) 1980. *The nutrient requirements of ruminant livestock.* Commonwealth Agricultural Bureaux, Slough.

Ahmed, W.M., Khadrawy, H.H., Hanafi, E.M.E., Hameed, A.R.A.E., Sabra, H.A. 2009. Effect of copper deficiency on ovarian activity in Egyptian buffalo-cows. *World Journal of Zoology,* 4, pp. 1-8.

Allen, J.D. and Gawthorne, J.M. 1987. Involvement of the solid phase of rumen digesta in the interaction between copper, molybdenum and sulphur in sheep. *British Journal of Nutrition*, 58, pp. 265-276.

Allen, M. and Oba, M. 1996. Fibre digestibility of forages. In: *Proceedings of the 57th Minnesota Nutritional Conference,* University of Minnesota, St Paul, pp. 151-171.

Alloway, B.J. 1973. Copper and molybdenum in swayback pastures. *Journal of Agricultural Science,* 80, pp. 521-524.

Alsemgeest, S.P.M., Taverne, M.A.M., Boosman, R., Weyden, B.C. and Gruys, E. 1993. Peripartum acute-phase protein serum amyloid-A concentration in plasma of cows and foetuses. *American Journal of Veterinary Research,* 54, pp. 164-167.

Andrew, S.M., Tyrrell, H.F., Reynolds, C.K. and Erdman, R.A. 1991. Net energy for lactation of calcium salts of long chain fatty acids for cows fed silage-based diets. *Journal of Dairy Science,* 74, pp. 2588-2600.

Animal Health and Veterinary Laboratories Agency (AHVLA). 2014. Yearly trends 2005–1012: Cattle. In: *Veterinary Investigation Surveillance Report (VIDA): 2012*. London. Available online from: https://www.gov.uk/government/publications/veterinary-investigation-diagnosis-analysis-vida-report-2012 [Accessed 8th October 2016].

Aschenbach, J.R., Kristensen, N.B., Donkin, S.S., Hammon, H.M. and Penner, G.B. 2010. Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *IUBMB Life,* 62, pp. 869-877.

Association of Official Analytical Chemists (AOAC) International. 2012. *Official Methods of Analysis.* 19th edition. AOAC International, Gaithersburg, USA.

Araya, M., Pizarro, F., Olivares, M., Arredondo, M., Gonzalez, and Mendez, M. 2006. Understanding copper homeostasis in humans and copper effects on health. *Biological Research,* 39, pp. 183-187.

Arthington, J.D. 2003. Copper antagonists in cattle nutrition. *Proceedings of the 14*th *Annual Florida Ruminant Nutrition Symposium*, Gainesville, Florida, pp. 48.

Arthington, J.D. 2005. Effects of copper oxide bolus administration or high-level copper supplementation on forage utilization and copper status in beef cattle. *Journal of Animal Science*, 83, pp. 2894-2900.

Arthington, J.D. and Spears, J.W. 2007. Effects of tribasic copper chloride versus copper sulfate provided in corn and molasses-based supplements on forage intake and copper status of beef heifers. *Journal of Animal Science,* 85, pp. 871-876.

Arthington, J.D., Spell, A.R., Corah, L.R. and Blecha, F. 1996. Effect of molybdenum-induced copper deficiency on in vivo and in vitro measures of neutrophil chemotaxis both before and following an inflammatory stressor. *Journal of Animal Science,* 74, pp. 22759-22764.

Atkins, N.E., Bleach, E.C.L and Sinclair, L.A. 2018. Periparturient and early lactation performance and metabolism of replacement Holstein-Friesian heifers out-wintered on fodder beet or perennial ryegrass compared with winter housing. *Grass and Forage Science,* 73, pp. 828-840.

Avramoglu, R.K. and Adeli, K. 2004. Hepatic regulation of apolipoprotein B. *Reviews in Endocrine and Metabolic Disorders,* 5, pp. 293-301.

Bach, A. 2011. Associations between several aspects of heifer development and dairy cow survivability to second lactation. *Journal of Dairy Science,* 94, pp. 1052-1057.

Bachrach, U. 1985. Copper amine oxidase and amines as regulators of cellular processes. In: *Structure and Functions of Amine Oxidases.* Edited Mondovi, B. CRC Press, Florida, USA.

Barry, T.N., Reid, T.C., Millar, K.R., and Sadler, W.A. 1981. Nutritional evaluation of kale (*Brassica oleracea)* diets. 2. Copper deficiency, thyroid function and selenium status in young cattle and sheep fed kale for prolonged periods. *Journal of Agricultural Science,* 96, pp. 269-282.

Bauchart, D. 1993. Lipid absorption and transport in ruminants. *Journal of Dairy Science,* 76, pp. 3864-3881.

Bauman, D.E. and Currie, W.B. 1980. Partitioning of nutrients during pregnancy and lactation: A review of mechanisms involving homoeostasis and homeorhesis. *Journal of Dairy Science,* 63, pp. 1514-1529.

Beckman, J.L. and Weiss, W.P. 2005. Nutrient digestibility of diets with different fibre to starch ratios when fed to lactating dairy cows. *Journal of Dairy Science,* 88, pp. 1015-1023.

Bellone, G., Aste-Amezaga, M., Trinchieri G. and Rodeck, U. 1995. Regulation of NK cell functions by TGF- β1. *Journal of immunology,* 1995, 155, pp. 1066-1073.

Bennets, H.W. and Hall, T.H.B. 1939. “Falling disease” of cattle in the south-west of Western Australia. *Australian Veterinary Journal,* 15, pp. 152-159.

Bidewell, C.A., David, G.P, and Livesey, C.T. 2000. Copper toxicity in cattle. *Veterinary Record,* 147, pp. 399-400.

Bidewell, C.A., Drew, J.R., Payne, J.H., Sayers, A.R., Higgins, R.J. and Livesey, C.T. 2012. Case study of copper poisoning in a British Dairy herd. *Veterinary Record,* 170, pp. 464-468.

Birben, E., Sahiner, U.M., Sackesen, C., Eerzurum, S., and Kalyaci, O. 2012. Oxidative Stress and Antioxidant Defence. *World Allergy Organization Journal,* 5 pp. 9-19.

Black, D.H. and French, N.P. 2004. Effects of three types of trace element supplementation on the fertility of three commercial dairy herds. *Veterinary Record: Journal of the British Veterinary Association,* 154, pp. 652-658.

Blakey, B.R. and Hamilton, D.L. 1985. Ceruloplasmin as an indicator of copper status in cattle and sheep. *Canadian Journal of Comparative Medicine,* 49, pp. 405-408.

Bradley, A.S., Leavitt, W.D., and Johnston, D.T. 2011. Revisiting the dissimilatory sulfate reduction pathway. *Geobiology,* 9, pp. 446-457.

Boila, R.J., Devlin, T.J., Drysdale, R.A. and Lillie, L.E. 1984. The severity of hypocupremia in selected herds of beef cattle in Northwestern Manitoba. *Canadian Journal of Animal Science,* 64, pp. 919-936.

Bonham, M., O'Connor, J.M., Hannigan, B.M. and Strain, J.J. 2002. The immune system as a physiological indicator of marginal copper status? *British Journal of Nutrition,* 87, pp. 393-403.

Borgstahl, G.E.O, Parge, H.E., Hickey, M.J., Johnston, M.J., Boissinot, M., Hallewell, R.A., Lepock, J.R, Cabelli, D.E. and Tainer, J.A. 1996. Human Mitochondrial Manganese Superoxide Dismutase Polymorphic Variant Ile58Thr Reduces Activity by Destabilizing the Tetrameric Interface. *Biochemistry,* 35, pp. 4287-4297.

Boulton, A.C., Rushton, J., and Wathes, D.C. 2015. The management and associated costs of rearing heifers on UK dairy farms from weaning to conception. *Open Journal of Animal Sciences,* 5, pp. 294-308.

Bourne, N., Swali, A., Jones, A.K., Potterton, S. and Wathes, D.C. 2007. The effects of size and age at first calving on subsequent fertility in dairy cows. In: *Reproduction in domestic ruminants 6.* Edited by Juengel, J.L., Murray, J.F. and Smith, M.F. Nottingham University Press, Nottingham, UK, pp. 526.

Boyne, R. and Arthur, J.R. 1981. Effects of selenium and copper deficiency on neutrophil function in cattle. *Journal of Comparative Pathology (UK),* 91, pp. 271-276.

Braselton, W.E., Stuart, K.J., Mullaney, T.P. and Herdt, T.H. 1997. Biopsy mineral analysis by inductively coupled plasma-atomic emission spectroscopy with ultrasonic nebulization. *Journal of Veterinary Diagnostic Investigation,* 9, pp. 395-400.

Bremner, I. 1993. In: *Proceedings of the 8th international symposium on trace elements in man and animals,* Gersdorf, Germany.

Bremner, I. and Dalgarno, A.C. 1973. Iron metabolism in the veal calf. 2. Iron requirements and the effect of copper supplementation. *British Journal of Nutrition,* 30, pp. 61-76.

Brockus, C.W. 2011. Erythrocytes. In: *Duncan and Prasse's veterinary laboratory medicine: clinical pathology.* 5th edition. Edited by Latimer, K.S. Wiley Publishing, Chichester, UK.

Broster, W.H. and Broster, V.J. 1998. Body condition score of dairy cows. *Journal of Dairy Research,* 65, pp. 155-173.

Brown, T.F. and Zeringue, L.K. 1994. Laboratory evaluations of solubility and structural integrity of complexed and chelated trace mineral supplements. *Journal of Dairy Science,* 77, pp.181-189.

Buckley, W.T. 1991. A kinetic model of copper metabolism in lactating dairy cows. *Canadian Journal of Animal Science,* 71, pp. 155-166.

Butler, W.R. 2001. Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. *Animal Science Occasional Publication,* 26, pp. 133-145.

Butler, W.R., Calaman, J.J. and Beam S.W. 1996. Plasma and milk urea nitrogen in relation to pregnancy rate in lactating dairy cattle. *Journal of Animal Science,* 74, pp. 858-865.

Butler, W.R. 2005. Nutrition, negative energy balance and fertility in the postpartum dairy cow. *Cattle Practice,* 13, pp. 13-18.

Caldeira, R.M., Belo, A.T., Santos, C.C., Vazques, M.I. and Portugal, A.V. 2007. The effect of body condition score on blood metabolites and hormonal profiles in ewes. *Small Ruminant Research,* 68, pp. 233-241.

Cannizzo, C., Gianesella, M., Giudice, E., Messina, V., Piccione, G. and Morgante, M. 2012. Serum acute phase proteins in cows with SARA (Subacute Ruminal Acidosis) suspect. *Brazilian Journal of Animal and Veterinary Science,* 64, pp. 15-22.

Carmo, C.A., Batistel, F., De Souza, F., Martinez, J.C., Correa, P., Pedrosa, A.M. and Santos, F.A.P. 2014. Starch levels on performance, milk composition and energy balance of lactating dairy cows. *Tropical Animal Health and Production,* 47, pp. 179-184.

Casper, D.P., Schingoethe, D.J. and Eisenbeisz, W.A. 1990. Response of early lactation dairy cows fed diets varying in source of nonstructural carbohydrate and crude protein. *Journal of Dairy Science,* 73, pp. 1039-1050.

Cao. X., Antonyuk, S.V., Seetharam, S.V., Whitson, L.J., Taylor, A.B., Holloway, S.P., Strange, R.W., Doucette, P.A., Valentine, J.S., Tiwari, A., Hayward, L.J., Padua, S., Cohlberg, J.A., Hasnain, S.S. and Hart, P.J. 2008. Structures of the G85R Variant of SOD1 in Familial Amyotrophic Lateral Sclerosis. *Journal of Biological Chemistry,* 283, pp. 169-177.

Castillo, A.R., St-Pierre, N.R., Silva del Rio, N. and Weiss, W.P. 2013. Mineral concentrations in diets, water, and milk and their value in estimating on-farm excretion of manure minerals in lactating dairy cows. *Journal of Dairy Science,* 96, pp. 3388-3398.

Ceroni, V., Turmalaj, L. and Duro, S. 2012. Haematological Indicators Affected by the Subacute Ruminal Acidosis Cows. *Journal of Animal and Veterinary Advances,* 11, pp. 927-930.

Chang, L.Y., Slot, J.W., Geuze, H.J. and Crapo, J.D. 1988. Molecular immunocytochemistry of the CuZn superoxide dismutase hepatocytes. *Journal of Cell Biology,* 107, pp. 2169-2179.

Chanjula, P., Wanapat, M., Wachirapakorn, C. and Rowlinson, P. 2004. Effect of synchronizing starch sources and protein (NPN) in the rumen on feed intake, rumen microbial fermentation, nutrient untilisation and performance of lactating dairy cows. *Asian Australian Journal of Animal Science,* 17, pp. 1400-1410.

Chang, I.C., Lee, T. and Matrone, G. 1975. Development of ceruloplasmin in pigs during the neonatal period. *Journal of Nutrition,* 105, pp. 624-630.

Chase, C., Beede, D., Van Horn, H., Shearer, J., Wilcox, C. and Donovan, G. 2000. Responses of lactating dairy cows to copper source, supplementation rate, and dietary antagonist (iron). *Journal of Dairy Science,* 83, pp. 1845-1852.

Chelly, J., Tümer, Z., Tønnesen, T., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N. and Monaco, A.P. 1993. Isolation of a candidate gene for menkes disease that encodes a potential heavy metal binding protein. *Nature Genetics,* 3, pp. 9-14.

Cherian, M.G. and Nordberg, M. 1983. Cellular adaptation in metal toxicology and metallothionein. *Toxicology,* 28, pp. 1-15.

Cho, Y.H., Jeong, D.W., Lee, S.Y., Park, S.K., Yoon, K.T., Kim, Y.J., Lee, J.K. and Lee, Y.H. 2011. A case of wilson's disease in patient with mildly elevated liver enzymes. *Korean Journal of Family Medicine*, 2011, 32, pp. 205-208.

Chooi, K.F., Hutagalung, R.I. and Mohamed, W.W. 1988. Copper toxicity in sheep fed oil palm by-products. *Australian Veterinary Journal,* 65, pp. 156-157.

Cisternas, F.A., Tapia, G., Arrendondo, G., Cartier-Ugarte, D., Romanque, D., Sierralta, W.D., Vial, M.T., Videla, L.A. and Arya, M. 2005. Early histological and functional effects of chronic copper exposure in rat liver. *Biometals,* 18, pp. 541-551.

Clarke, N.J. and Laurie, S.H. 1980. The copper-molybdenum antagonism in ruminants. 1. Formation of thiomolybdates in animal rumen. *Journal of Inorganic Biochemistry,* 12, pp. 37-43.

Collins, J.F., Prohaska, J.R. and Knutson, M.D. 2010. Metabolic crossroads of iron and copper. *Nutrition Reviews,* 68, pp. 133-147.

Commonwealth Scientific and Industrial Research Organisation (CSIRO). 2007. *Nutrient requirements of domesticated ruminants.* CSIRO Publishing, Australia, Collingwood.

Cope, C.M, Mackenzie, A.M., Wilde, D. and Sinclair, L.A. 2009. Effects of level and form of dietary zinc on dairy cow performance and health. *Journal of Dairy Science,* 92, pp. 2128-2135.

Corbett, W.S., Saylor, W.W., Long, T.A. and Leach, R.M., Jr. 1978. Intracellular distribution of hepatic copper in normal and copper-loaded sheep. *Journal of Animal Science,* 47, pp. 1174-1179.

Corl, B.A., Lacy, S.H., Baumgard, L.A., Dayer, D.A., Griinari, J.M., Phillips, B.S. and Bauman, D.E. 1999. Examination of the importance of D9 -desaturase and endogenous

synthesis of CLA in lactating dairy cows. *Journal of Animal Science,* 77 (Supplement 1), pp. 118 (Abstract).

Cousins, R.J. 1985. Absorption, transport, and hepatic metabolism of copper and zinc: special reference to metallothionein and ceruloplasmin. *Physiological Reviews,* 65, pp. 238-309.

Cummins, L.J. and Harris, D.J. 1984. Temporary infertility associated with parental copper therapy in cattle. *Australian Veterinary Journal,* 61, pp. 164-165.

Cundy, T.F., Butler, J., Pope, R.M., Saggar-Malik, A.K., Wheeler, M.J. and Williams, R. 1991. Amenorrhoea in women with non-alcoholic chronic liver disease. *International Journal in Gastroenterology,* 32, pp. 202-206.

Cunningham, I.J. 1950. Copper and molybdenum in relation do diseases of cattle and sheep in New Zealand. In: *Copper Metabolism: A Symposium on Animal, Plant, and Soil Relationships.* Edited by McElroy, W.D. and Glass, B. Johns Hopkins Press, Baltimore, USA, pp. 246-273.

Członkowska, A. and Milewski, B. 1976. Immunological observations on patients with wilson's disease. *Journal of the Neurological Sciences,* 29, pp. 411-421.

Dalsgaard, K., Hilgers, L. and Trouve, G. 1990. Classical and new approaches to adjuvant use in domestic food animals. In: *Immunomodulation in Domestic Food Animals.* Edited by Blecha, F. and Charley, B. Academic Press Inc, San Diego, USA, 35, pp. 121-160.

Das, S., Levinson, B., Vulpe, C., Whitney, S., Gitschier, J. and Packman, S. 1995. Similar splicing mutations of the Menkes/mottled copper-transporting ATPase gene in occipital horn syndrome and the blotchy mouse. *American Journal of Human Genetics,* 56, pp. 570-576.

Davies, D.C. and Jebbett, I.H. 1981. Liver biopsy of cattle. *In Practice,* 3, pp. 14-16.

Davis, C.D. 2003. Low dietary copper increases fecal free radical production, fecal water alkaline phosphatase activity and cytotoxicity in healthy men. *Journal of Nutrition,* 133, pp. 522-527.

Davis, G.K. and Mertz, W. 1987. *Trace Elements in Human and Animal Nutrition.* Edited by Mertz, W. Academic Press, London, UK.

Day, M.L., Imakawa, K., Garcia-Winder, M., Zalesky, D.D., Schanbacher, B.D., Kittoc, R.J. and Kinder, J.E. 1984. Endocrine mechanisms of puberty in heifers. Estradiol negative feed back regulation of luteinizing hormone secretion. *Biology Reproduction,* 31, pp. 332.

Del Rincon, J.P., Iida, K., Gaylinn, B.D., McCurdy, C.E., Leitner, J.W., Barbour, L.A., Kopchick, J.J., Friedman, J.E., Draznin, B. and Thorner, M.O. 2007. Growth hormone regulation of p85 alpha expression and phosphoinositide 3-kinase activity in adipose tissue: mechanism for growth-mediated insulin resistance. *Diabetes,* 56, pp. 1638-1646.

Delfiol, D.J., Cagnini, D.Q., Cunha, P.H., Crosignani, N., Wouters, A.T., Wouters, F., Driemeier, D. and Borges, A.S. 2013. Clinical and laboratory aspects of sheep supplemented with high levels of sulfur in diet to induce polioencephalomalacia. *Pesquisa Veterinária Brasileira,* 33, pp. 435-442.

Dennis, T.S., Suarez-Mena, F.X., Hu, W., Hill, T.M., Quigley, J.D. and Schlotterbeck, R.L. 2019. Effects of milk replacer feeding rate and long-term antibiotic inclusion in milk replacer on performance and nutrient digestibility of Holstein dairy calves up to 4 months of age. *Journal of Dairy Science,* 102, pp. 2094-2102.

Dias, R. S., López, S., Montanholi, Y.R., Smith, B., Haas, L.S., Miller, S.P. and France, J. 2013. A meta-analysis of the effects of dietary copper, molybdenum, and sulfur on plasma and liver copper, weight gain and feed conversion in growing-finishing cattle. *Journal of Animal Science,* 91, pp. 5714-5723.

Dick, A.T. 1954. Studies on the assimilation and storage of copper in crossbred sheep. *Australian Journal of Agricultural Research,* 5, pp. 511-555.

Dick, A.T., Dewey, D.W. and Gawthorne, J.M. 1975. Thiomolybdates and the copper-molybdenum-sulphur interaction in ruminant nutrition. *The Journal of Agricultural Science,* 85, pp. 567-568.

Dirksen, G. 1985. The rumen acidosis complex- Recent knowledge and experiences (1): A review. *Tierarztliche Praxis,* 13, pp. 501-512.

Drackley, J.K. 1999. Biology of dairy cows during the transition period: the final frontier. *Journal of Dairy Science,* 82, pp. 2259-2273.

Drewnoski, M.E. and Hansen, S.L. 2013. Effect of delaying the feeding of high sulfur until 28 days after adaptation to finishing diet on cattle intake, gain, and ruminal hydrogen sulfide concentrations. *Livestock Science,* 155, pp. 230-235.

Drewnoski, M.E., Ensley, S.M., Beitz, D.C., Schoonmaker, J.P., Loy, D.D., Imerman, P.M., Rathje, J.A. and Hansen, S.L. 2012. Assessment of ruminal hydrogen sulfide or urine thiosulfate as diagnostic tools for sulfur induced polioencephalomalacia in cattle. *Journal of Veterinary Diagnostic Investigation,* 24, pp. 702-709.

Drewnoski, M.E., Pogge, D.J. and Hansen, S.L. 2014a. High-sulfur in beef cattle diets: A review. *Journal of Animal Science,* 92, pp. 3763-3780.

Drewnoski, M.E., Doane, P. and Hansen, S.L. 2014b. Ferric citrate decreases ruminal hydrogen sulphide concentrations in feedlot cattle fed diets high in sulphate. *British Journal of Nutrition,* 111, pp. 261-269.

Dougherty, R.W. and Cook, H.M. 1962. Routes of eructated gas expulsion in cattle- A quantitative study. *American Journal of Veterinary Research,* 23, pp. 997-1000.

Du, Z., Hemken, R.W. and Harmon, R.J. 1996. Copper metabolism of holstein and jersey cows and heifers fed diets high in cupric sulfate or copper proteinate. *Journal of Dairy Science,* 79, pp. 1873-1880.

Du, X., Chen, L., Huang, D., Peng, Z., Zhao, C., Zhang, Y., Wang, Z., Li, X. and Liu, G. 2017. Elevated apoptosis in the liver of dairy cows with ketosis. *Cellular Physiology and Biochemistry,* 43, pp. 568-578.

Du Plessis, S.S., van Niekerk, F.E. and Coetzer, W.A. 1999. The effect of dietary molybdenum and sulphate on the oestrus cycle and ovulation in ewes after manipulation with exogenous progesterone alone or in combination with FSH and LH. *Small Ruminant Research,* 33, pp. 71-76.

Durand, M. and Kawashima, R. 1980. Influence of minerals in rumen microbial digestion. In: *Digestive physiology and metabolism in ruminants.* Edited by Ruckebisch, Y. and Thivend, P. Avi Publishing Company, New York, USA, pp. 375-408.

Engle, T.E. 2014. Copper and lipid metabolism in beef cattle: A review. *Journal of Animal Science*, 89, pp. 591-596.

Engle, T. and Spears, J. 2000. Effects of dietary copper concentration and source on performance and copper status of growing and finishing steers. *Journal of Animal Science,* 78, pp. 2446-2451.

Engle, T. E., Spears, J. W., Fellner, V. and Odle, J. 2000. Effects of soybean oil and dietary copper on ruminal and tissue lipid metabolism in finishing steers. *Journal of Animal Science,* 78, pp. 2713-2721.

Engle, T.E., Fellner, V. and Spears, J.W. 2001. Copper status, serum cholesterol, and milk fatty acid profile in Holstein cows fed varying concentrations of copper. *Journal of Dairy Science,* 84, pp. 2308-2313.

Essig, H.W., Davis, J.D. and Smithson, L.J. 1972. Copper sulfate in steer rations. *Journal of Animal Science,* 35 pp. 436-439.

Evans, G.W. 1973. Copper homeostasis in the mammalian system. *Physiological Reviews,* 53, pp. 535-570.

Fell, B.F., Farmer, L.J., Farquharson, C., Bremner, I., and Graca, D.S. 1985. Observations on the pancreas of cattle deficient in copper. *Journal of Comparative Pathology,* 95, pp. 573-590.

Ferguson, J.D., Galligan, D.T. and Thomenson, N. 1994. Principal descriptors of body condition score in Holstein cows. *Journal of Dairy Science,* 77, pp. 2695-2703.

Firacative, C., Gressler, A.E., Schubert, K., Schulze, B. Müller, U., Brombacher, F., von Bergen, M. and Alber, G. 2018. Identification of T helper (Th)1- and Th2- associated antigens of *Cryptococcus neoformans* in a murine model of pulmonary infection. *Sientific Reports,* 8, pp. 2681-2694.

Firkins, J. 1997. Effect of physical processing of corn silage and grain. In: *Proceedings of the Tri-State Dairy Nutrition Conference.* The Ohio State University, Columbus, United States of America.

Fleming, R. and Gitlin, J.D. 1990. Primary structure of rat ceruloplasmin and analysis of tissue-specific gene expression during development. *Journal of Biological Chemistry,* 265, pp. 7701-7707.

Foldager, J., Sejrsen, K. and Sørenson, J.T. 1988. The effect of plane of nutrition on growth and feed utilization in RDM and SDM heifers- revision of energy requirements for growth. *Report from the Nutritional Institute of Animal Science,* National Institute of Animal Science, Tjele, Denmark, 693.

Foster, D.L. and Ryan, K.D. 1981. Endocrine mechanisms governing transition into adulthood in sheep. *Journal of Reproduction and Fertility,* 30, pp. 75-90.

Fridovich, I. 1975. Superoxide dismutases. *Annual Reviews of Biochemistry,* 44, pp. 147-159.

Fry, R.S. 2011. *Dietary and genetic effects on cellular copper homeostasis in bovine and porcine tissues.* Ph.D. Thesis, North Carolina State University.

Fuentealba, I.C., Davis, R.W., Elmes, M.E., Jasani, B. and Haywood, S. Mechanisms of tolerance in the copper-loaded rat liver. *Experimental and Molecular Pathology,* 59, pp. 71-84.

Fukai, T. and Ushio-Fukai, M. 2011. *Superoxide Dismutases: Role in Redox Signaling, Vascular Function, and Diseases. Antioxidants and Redox Signaling,* 15, pp. 1583-1606.

Fukuda, H., Ebara, M., Yamada, H., Arimoto, M., Okabe, S., Obu, M., Yoshikawa, M., Sugiura, N. and Saisho, H. 2007. Trace elements and cancer. *JMAJ - Japan Medical Association Journal,* 47, pp. 391-395.

Gajewshi, T.F. and Fitch, F.W. 1988. Anti-proliferative effect of IFN-gamma in immune regulation. 1. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *Journal of Immunology,* 140, pp. 4245-4252.

Gao, F. Kinnula, V.L., Myllarniemi, M. and Oury, T.D. 2008. Extracellular superoxide dismutase in pulmonary fibrosis. *Antioxidant Redox Signalling,* 10, pp. 343-354.

Gault, M.H., Stein, J. and Arnoff, A. 1966. Serum ceruloplasmin in hepatobiliary and other disorders: significance of abnormal values. *Gastroenterology,* 50, pp. 8-18.

Garnsworthy, P.C. and Topps, J.H. 1982. The effect of body condition of dairy cows at calving on their food intake and performance when given complete diets. *Animal Production,* 35, pp. 113-119.

Gengelbach, G.P. 1994. *Effects of copper deficiency on cellular immunity in cattle.* Raleigh: North Carolina State University.

Gengelbach, G.P., Ward, J.D, and Spears J.W. 1994. Effects of dietary copper, iron and molybdenum on growth and copper status of beef cows and calves. *Journal of Animal Science,* 72, pp. 2722-2727.

Georgievskii, V.I., Annenkov, B.N. and Samokhin, V.T. 1982. *Mineral nutrition of animals: Studies in the Agricultural and Food Sciences,* Butterworth, London.

Giannini, E.G., Testa, R. and Savarino, V. 2005. Liver enzyme alteration: a guide for clinicians. *Review Synthèse,* 172, pp. 367-379.

Gitlin, J.D. and Janeway, C.A. 1960. Turnover of the copper and protein moieties of ceruloplasmin. *Nature,* 186, pp. 693.

Gitlin, J.D., Schroeder, J.J., Lee-Ambrose, L.W. and Cousins, R.J. 1992. Mechanisms of caeruloplasmin biosynthesis in normal and copper-deficient rats. *Biochemistry Journal,* 282, pp. 835-839.

Gómez L.M., Posada, S.L. and Olivera, M. 2016. Starch in ruminant diets: a review. *Revista Colombiana de Ciencias Pecuarias* 29, pp. 77-90.

Gong, J.G. 2002. Influences of metabolic hormones and nutrition on ovarian follicle development in cattle: practical implications. *Domestic Animal Endocrinology,* 23, pp. 229-241.

Gooneratne, S.R., Howell, J.M. and Gawthorne, J. 1979. Intracellular distribution of copper in the liver of normal and copper loaded sheep. *Research in Veterinary Science,* 27, pp. 30-66.

Gooneratne, S.R., Symonds, H.W., Bailey, J.V. and Christensen, D.A. 1994. Effects of dietary copper, molybdenum and sulfur on biliary copper and zinc excretion in simmental and angus cattle. *Canadian Journal of Animal Science,* 74, pp. 315-325.

Goselink, R.M.A. and Jongbloed, A.W. 2012. *Zinc and copper in dairy cattle feeding.* Wageningen UR Livestock Research, Lelystad.

Gould, D.H. 1998. Polioencephalomalacia. *Journal of Animal Science,* 76, pp. 309-314.

Gould, L. and Kendall, N.R. 2011. Role of the rumen in copper and thiomolybdate absorption. *Nutrition Research Reviews,* 24, pp. 176-182.

Gould, D.H., Cummings, B.A. and Hamar, D.W. 1997. In vivo indicators of pathologic ruminal sulfide production in steers with diet-induced polioencephalomalacia. *Journal of Veterinary Diagnostic Investigation,* 9, pp.72-76.

Grace, N.D. and Wilson, P.R. 2002. Trace element metabolism, dietary requirements, diagnosis and prevention of deficiencies in deer. *New Zealand Veterinary Journal,* 50, pp. 252-259.

Grummer, R.R. and Carroll, D.J. 1991. Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. *Journal of Animal Science (USA)*, 69, pp. 3838-3852.

Gu, K., Xuekui, L., Xiang, W. and Jiang, X. 2019. The relationship between serum copper and overweight/obesity: a meta-analysis. *Biological Trace Element Research.*

Haber, M.M., West, A.B., Haber, A.D. and Reuben, A. 1995. Relationship of aminotransferases to liver histological status in chronic hepatitis. *American Journal of Gastroenterology,* 90, pp. 1250-1257.

Habib, S.A., Saad, E.A., Elsharkawy, A.A., and Attia, Z.R. 2015. Proinflammatory adipocytokines, oxidative stress, insulin, Zn and Cu: interrelations with obesity in Egyptian non-diabetic obese children and adolescents. *Advances in Medical Science,* 60, pp. 179-185.

Hansen, S.L., Schlegel, P., Legleiter, L.R., Lloyd, K.E., and Spears, J.W. 2008. Bioavailability of copper from copper glycinate in steers fed high sulfur and molybdenum. *Journal of Animal Science,* 86, pp. 173-179.

Hansen, S.L., Ashwell, M.S., Legleiter, L.R., Fry, R.S., Lloyd, K.E., and Spears, J.W. 2009. The addition of high manganese to a copper-deficient diet further depresses copper status and growth of cattle. *British Journal of Nutrition,* 101, pp. 1068-1078.

Hansen, S.L., Trakooljul, N., Liu, H.C.S., Hicks, J.A., Ashwell, M.S. and Spears, J.W. 2010. Proteins involved in iron metabolism in beef cattle are affected by copper deficiency in combination with high dietary manganese, but not by copper deficiency alone. *Journal of Dairy Science*, 88, pp. 275-283.

Hardman, B., Michalczyk, A., Greenough, M., Camakaris, J., Mercer, J.F.B. and Ackland, M.L. 2007. Hormonal regulation of the menkes and wilson copper-transporting ATPases in human placental jeg-3 cells. *Biochemical Journal*, 402, pp. 241-250.

Harris, E.D. 2000. Cellular copper transport and metabolism. *Annual Review of Nutrition,* 20, pp. 291-310.

Hassanat, F., Gervais, R., Julien, C., Massé, D.I., Lettat, A., Chouinard, P.Y., Petit, H.V. and Benchaar, C. 2014. Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. *Journal of Dairy Science,* 96, pp. 4553-4567.

Hayden, M.S. and ghosh, S. Shared principles in NF-kappaB signaling. *Genes and Development,* 132, pp. 344-362.

Hawkins, D. 2014. Effects of subcutaneously injected Ca Cu EDTA on concentrations of Cu in liver, milk production and reproductive performance in New Zealand dairy cows. *New Zealand Veterinary Journal,* 62, pp. 244-249.

Healey, C.J., Chapamn, R.W. and Fleming, K.A. 1995. Liver histology in hepatitis C infection: a comparison between patients with persistently normal or abnormal transaminases. *Gut,* pp. 274-278.

Hellman, N.E. and Gitlin, J.D. 2002. Ceruloplasmin metabolism and function. *Annual Review of Nutrition,* 22, pp. 439-458.

Hennessy, D.W. and Nolan, J.V. 1988. Nitrogen kinetics in cattle fed a mature subtropical grass hay with and without protein meal supplementation. *Australian Journal of Agricultural Research,* 39, pp. 1135-1150.

Henry, R.J., Cannon, D.C. and Winkleman, J.W. 1974. *Clinical chemistry: Principles and techniques.* Harper and Row Publishers, London, UK.

Herdt, T.H. 2000. Ruminant adaptation to negative energy balance: Influences on the etiology of ketosis and fatty liver. *Veterinary Clinics of North America: Food Animal Practice,* 16, pp. 215-230.

Hernandez, S., Tsuchiva, Y., García-Ruiz, J.P., Lalioto, V., Nielsen, S., Cassio, D. and Sandoval, I.V. 2008. ATP7B copper-regulated traffic and association with the tight junctions: copper excretion into the bile. *Gastroenterology,* 134, pp. 1215-1223.

Hills, J.L., Wales, W.J., Dunshea, F.R., Garcia, S.C. and Roche, J.R. 2015. Invited Review: An evaluation of the likely effects of individualised feeding of concentrate supplements to pasture-based dairy cows. *Journal of Dairy Science,* 98, pp. 1363-1401.

Hoffman, P.C., Weigel, K.A. and Wernberg, R.M. 2008. Evaluation of equations to predict dry matter intake of dairy heifers. *Journal of Dairy Science,* 91, pp. 3699-3709.

Hodzic, A., Zuko, A., Avdic, R., Alic, A., Omeragic, J. and Jazic, A. 2013. Influence of *fasciola hepatica* on serum biochemical parameters and vascular and biliary system of sheep liver, *Iranian Journal of Parasitology,* 8, pp. 92-98.

Holtzman, N.A. and Gaumnitz, B.M. 1970. Studies on the rate of release and turnover of ceruloplasmin and apoceruloplasmin in rat plasma. *Journal of Biological Chemistry,* 245, pp.2354-2358.

Horn, D. and Barrientos, A. 2008. Mitochondrial copper metabolism and delivery to cytochrome c oxidase. *IUBMB Life,* 60, pp. 421-429.

Howell, J.M. and Gooneratne, S.R. 1987. The pathology of copper toxicity in animals. In: *Copper in Animals and Man*. Edited by Howell, J.M. and Gawthorne, S.R. CRC Press, Florida, pp. 53-78.

Howie, N. 2017. *Veterinary surgeon and livestock production consultant.* Personal Communication.

Humann-Ziehank, E., Coenen, M., Ganter, M. and Bickhardt, K. 2001. Long-term observation of subclinical chronic copper poisoning in two sheep breeds. *Journal of Veterinary Medicine Series A,* 48, pp. 429-439.

Humer, E., Petri, M., Aschenbach, J.R., B.J. Bradford, Penner, G.B., Tafaj, M., Südekum, and Zebeli, Q. 2018. Invited Review: Practical feeding management recommendations to mitigate the risk of subacute ruminal acidosis in dairy cattle. *Journal of Dairy Science,* 101, pp. 872-888.

Humphries, W.R., Phillippo, M., Young, B.W. and Bremner, I. 1983. The influence of dietary iron and molybdenum on copper metabolism in calves. *British Journal of Nutrition,* 49, pp. 77-86.

Hunter, A.G., Suttle, N.F., Martineau, H.M., Spence, M.A., Thomson, J.R., Macrae, A.I. and Brown, S. 2013. Mortality, hepatopathy and liver copper concentrations in artificially reared jersey calves before and after reductions in copper supplementation. *Veterinary Record,* 172, pp. 46-51.

Hussein, A.A. 2017. *The Effects of Forage Type and Antagonists on Copper Metabolism in Sheep.* Ph.D. Thesis, Harper Adams University.

The International Organisation for Standardisation (ISO). 2000. 6493- Animal feeding stuffs- Determination of starch content- Polarimetric method, 1, pp. 1-10.

Ingenhoff, L., Hall, E., Ranjbar, N.S. and House, J.K. 2017. Effect of insemination site and diameter of the pre-ovulatory follicle on the odds of pregnancy in heifers using sexed or non-sexed semen. *Australian Veterinary Journal,* 95, pp. 317-324.

Ivan, M., Hidiroglou, M., Al-Ismaily, S.I, Al-Sumry, H.S. and Harper R.B. 1990. Copper deficiency and posterior analysis (Shalal) in small ruminants in the Sultanante of Oman. *Tropical Animal Health and Production,* 22, pp. 217-225.

Jacklin, D. 2016. Cattle Nutrition: Excess dietary copper. *Veterinary Record*, 178, p. 123.

Jeffery, H.B. and Berg R.T. 1972. An evaluation of several measurements of beef cow size as related to progeny performance. *Canadian Journal of Animal Science,* 52, pp. 23-37.

Jesse, B.W., Emery, R.S. and Thomas, J.W. 1986. Control of bovine hepatic fatty acid oxidation. *Journal of Dairy Science,* 69, pp. 2298-2303.

Johnston, J.L. 1997. Molybdenum. In: *Handbook of Nutritionally Essential Mineral Elements.* Edited by O'Dell, B.L. and Unde, R.A.S., Marcel Dekker, New York, pp. 413-438.

Johnston, D.E. 1999. Special considerations in interpreting liver function tests. *American Family Physician,* 59, pp. 2223-2230.

Johnston, H., Beasley, L. and MacPherson, N. 2014. Copper toxicity in a New Zealand dairy herd. *Veterinary Ireland Journal,* 4, pp. 1-6.

Jolly, P.D., Clark, R.G., Fraser, A.J. 1987. Effect of copper status and copper supplementation on milk production and fertility in dairy cattle. In: *Proceedings of the 4th Asian Australian Animal Production Conference.* Edited by Organising Committee 4th AAP Congress.

Jones, D.G. and Suttle, N.F. 1987. Copper and disease resistance. In: *Trace Substances in Environmental Health- XXI.* University of Missouri, Missouri, pp. 514-525.

Jones, M.L. and Allison, R.W. 2007. Evaluation of the ruminant complete blood cell count. *Veterinary Clinics of North America: Food and Animal Practice,* 23, pp. 377-402.

Jurjanz, S., Colin-Schoellen, O., Gardeur, J.N. and Laurent, F. 1998. Alteration of milk fat by variation in the source and amount of starch in a total mixed diet to dairy cows. *Journal of Dairy Science,* 81, pp. 2924-2933.

Kalinowski, D.S., Stefani, C., Toyokuni, S., Ganz, T., Anderson, G.J., Subramaniam, N.V., Trinder, D., Olynyk, J.K., Chua, A., Jansson, P.J., Sahni, S., Lane, D.J.R., Merlot, A.M., Kovacevic, Z., Huang, M.L.H., Lee, C.S. and Richardson, D.R. 2016. Review: Redox cycling metals: Pedaling their roles in metabolism and their use in the development of novel therapeutics. *BBA - Molecular Cell Research,* 1863, pp. 727-748.

Kaya, S., Merhan, O., Kacar, C., Colak, A., and Bozukluhan, K. 2016. Determination of ceruloplasmin, some other acute phase proteins, and biochemical parameters in cows with endometritis. *Veterinary World,* 9, pp. 1056-1062.

Ke., Y and Symonds, H.W. 1989. Enhancement of tetrathiomolybdate induced biliary copper excretion in sheep by general anaesthesia and the effect on copper excretion in urine and bile. *Research in Veterinary Science,* 46, pp. 344-348.

Kegley, E.B. and Spears, J.W. 1994. Bioavailability of feed-grade copper sources (oxide, sulfate, or lysine) in growing cattle. *Journal of Animal Science,* 72, pp. 2728-2734.

Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Dernyck, R., Sporn, M.B. and Fauci, A.S. 1986. Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. *Journal of Experimental Medicine,* 163, pp. 1037-1050.

Kelleher, C.A. and Mason, J. 1986. Reversible inhibition of ovine caeruloplasmin by thiomolybdates. *International Journal of Biochemistry,* 18, pp. 629-635.

Kendall, N.R., Holmes-Pavord, H.R., Bone, P.A., Ander, E.L. and Young, S.D. 2015. Liver copper concentrations in cull cattle in the UK: Are cattle being copper loaded? *Veterinary Record: Journal of the British Veterinary Association,* 177, pp. 493-496.

Keunen, J.E., Plaizier, J.C., Kyriazakis, L., Duffield, T.F., Widowski, T.M., Lindinger, M.I., and McBride, B.W. 2002. Effects of subacute ruminal acidosis model on the diet selection of dairy cows. *Journal of Dairy Science,* 85, pp. 3304-3313.

Khan, M.A., Bach, A., Weary, D.M. and von Keyserlingk, M.A.G. 2016. Invited Review: Transitioning from milk to solid feed in dairy heifers. *Journal of Dairy Science,* 99, pp. 885-902.

Kidane, T.Z., Farhad, R., Lee, K.J., Santos, A., Russo, E. and Linder, M.C. 2012. Uptake of copper from plasma proteins in cells where expression of CTR1 has been modulated. *Biometals,* 25, pp. 697-709.

Kim, B.E., Nevitt, T. and Thiele, D.J. 2008. Mechanisms for copper acquisition, distribution and regulation. *Nature Chemical Biology,* 4, pp. 176-185.

Klomp, L.W.J., Farhangazi, Z.S., Dugan, L.L. and Gitlin, J.D. 1996. Ceruloplasmin gene expression in the murine central nervous system. *Journal of Clinical Investigation,* 98, pp. 207-215.

Kodama, H., Abe, T., Takama, M., Takahashi, I., Kodama, M. and Nishimura, M. 1993. Histochemical localization of copper in the intestine and kidney of macular mice: Light and electron microscopic study. *Journal of Histochemistry and Cytochemistry,* 41, pp. 1529-1535.

Krause, K.M., Combs, D.K., and Beauchemin, K.A. 2003. Effects of increasing levels of refined cornstarch in the diet of lactating dairy cows on performance and ruminal pH. *Journal of Dairy Science,* 86, pp. 1341-1353.

Krehbiel, C.R., Stock, R.A., Shain, D.H., Richards, C.J., Ham, G.A., McCoy, R.A., Klopfenstein, T.J., Britton, R.A. and Huffman, R.P. 1995. Effect of level and type of fat on subacute acidosis in cattle fed dry-rolled corn finishing diets. *Journal of Animal Science,* 73, pp. 2438-2446.

Kudrin, A.V. 2000. Trace elements in regulation of NF-kappaB activity. *Journal of Trace Elements in Medical Biology,* 14, pp. 129-142.

Kumaratilake, J.S. and Howell, J.M. 1987. Histochemical study of the accumulation of copper in the liver of sheep. *Research Veterinary Science,* 42, pp. 73-81.

Kumaratilake, J.S. and Howell, J.M. 1989. Intracellular distribution of copper in the liver of copper-loaded sheep- a subcellular fractionation study. *Journal of Comparative Pathology (UK),* 101, pp. 161-176.

KW Alternative Feeds. 2019. Product specification datasheets. Accessed online: https://www.kwalternativefeeds.co.uk/ [accessed 7th August 2019].

La Fontaine, S., Ackland, M.L. and Mercer, J.F. 2010. Mammalian copper-transporting P-type ATPases, ATP7A and ATP7B: emerging roles. *The international Journal of Biochemistry and Cell Biology,* 42, pp. 206-209.

Langlands, J.P., Donald, G.E., Bowles, J.E. and Smith, A.J. 1989. Trace element nutrition of grazing ruminants. 3. Copper oxide powder as a copper supplement. *Australian Journal of Agricultural Research,* 40, pp. 187.

Lannon, B. and Mason, J. 1986. The inhibition of bovine ceruloplasmin oxidase activity by thiomolybdates *in vivo* and *in vitro:* A reversible interaction. *Journal of Inorganic Biochemistry,* 26, pp. 107-115.

Laven R.A. and Livesey, C.T. 2004. The diagnosis of copper related disease: do we have the necessary tools? Part 1: Prevalence of disease and the diagnosis of copper toxicity. *Cattle Practice,* pp. 265-269.

Laven, R.A. and Livesey, C.T. 2005. The diagnosis of copper related disease, part 2: Copper repoonsive disorders. *Cattle Practice,* 13, pp. 55-60.

Laven, R.A., Lawerence, K.E. and Livesey, C.T. 2011. The assessment of blood copper status in cattle: A comparison of measurements of caeruloplasmin and elemental copper in serum and plasma. *New Zealand Veterinary Journal,* 55, pp. 171-176.

Leary, S.C., Kaufman, B.A., Pellecchia, G., Guercin, G.H., Mattman, A., Jaksch, M. and Shoubridge, E.A. 2004. Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. *Human Molecular Genetics,* 13, pp. 1839-1848.

Leaver, J.D. 1977. Rearing of dairy cattle. 7. Effect of level of nutrition and body condition on the fertility of heifers. *Animal Production,* 25, pp. 219-224.

Lee, J., Petris, M.J. and Thiele, D.J. 2002. Characterization of mouse embryonic cells deficient in the Ctr1 high affinity copper transporter - identification of a Ctr1-independent copper transport system. *Journal of Biological Chemistry,* 277, pp. 40253-40259.

Legleiter, L.R. and Spears, J.W. 2007. Plasma diamine oxidase: a biomarker of copper deficiency in the bovine. *Journal of Animal Science,* 85, pp. 2198-2204.

Lei, L., Xiaoyi, S. and Fuchang, L. Effect of dietary copper addition on lipid metabolism in rabbits. *Food and Nutrition Research,* 61, pp. 1-8.

Lenartowicz, M. and Krzeptowski, W. 2010. Structure and function of ATP7A and ATP7B proteins cu-transporting ATPases. *Postepy Biochemii,* 56, pp. 317-327.

Liesman, J.S., McNamara, J.P., Capuco, A.V., Binelli, M., Vanderkool, W.K., Emery, R.S., Tucker, H.A. and Moseley, W.M. 1995. Comparison of growth hormone-releasing factor and somatropin: Lipid and glucose metabolism in dairy cows. *Journal of Dairy Science,* 78, pp. 2159-2166.

Lilienbaum, A. and Israël, A. 2003. From calcium to NF-kappaB signaling pathways in neurons. *Molecular and Cellular Biology,* 23, pp. 2680-2698.

Linder, M.C. 1991. Introduction and overview of copper as an element essential for life. In: *Biochemistry of Copper.* Plenum Press, New York, pp. 1-13.

Linder, M.C. and Hazegh-Azam, M. 1996. Copper biochemistry and molecular biology. *American Journal of Clinical Nutrition,* 63, pp. 797S.

Lippard, S.J. and Berg, J.M. 1994. Principles of bioinorganic chemistry. University Science Books, Mill Valley, California, USA.

Little, M.W., O'Connell, N.E. and Ferris, C.P. 2016. A comparison of individual cow versus group concentrate allocation strategies on dry matter intake, milk production, tissue changes, and fertility of Holstein-Friesian cows offered a grass silage diet. *Journal of Dairy Science,* 99, pp. 4360-4373.

Liu, N., Lo, L.S., Askary, S.H., Jones, L., Kidane, T.Z., Trang, T., Nguyen, M., Goforth, J., Chu, Y.H., Vivas, E., Tsai, M., Westbrook, T. and Linder, M.C. 2007. Transcuprein is a macroglobulin regulated by copper and iron availability. *Journal of Nutritional Biochemistry,* 18, pp. 597-608.

Livesey, C.Y., Bidewell, C.A., Crawshaw, T.R. and David, G.P. 2002. Investigation of copper poisoning in adult cows by the veterinary laboratories agency. *Cattle Practice,* 10, pp. 289-294.

López-Alonso, M., Prieto, F., Miranda, M., Castillo, C., Hernández, J.R. and Benedito, J.L. 2005. Intracellular distribution of copper and zinc in the liver of copper-exposed cattle from northwest Spain. *The Veterinary Journal,* 170, pp. 332-338.

Lopex-Guisa, J.M. and Satter, L.D. 1992. Effect of copper and cobalt addition on digestion and growth in heifers fed diets containing alfalfa silage or corn crop residues. *Journal of Dairy Science,* 75, pp. 247-256.

Lord, J.D., McIntosh, B.C., Greenebrg, P.D. and Nelson, B.H. 2000. The IL-2 receptor promotes lymphocyte proliferation and induction of the *c-myc*, *bcl-2*, and *bcl-x* genes through the trans-activation domain of stat5. *Journal of Immunology,* 164, pp. 2533-2541.

Lu, W.Q., Qiu, J.L., Huang, Z.L. and Liu, H.Y. 2016. Enhanced circulating transforming growth factor beta 1 is causally associated with an increased risk of hepatocellular carcinoma: a mendelian randomization meta-analysis. *Oncotarget,* 7, pp. 84695-84704.

Lucas, H.L. 1960. Critical features of good dairy feeding experiments. *Journal of Dairy Science,* 43, pp. 193-212.

Lutnicki, K., Madej, E., Riha, T. and Kurek, L. 2014. Polioencephalomalacia in ruminants caused by excessive amount of sulphur- a review. *Bulletin of the Veterinary Institute in Pulawy,* 58, pp. 321-326.

Lutsenko, S., Barnes, N., Bartee, M. and Dmitriev, O. 2002. Function and regulation of human copper-transporting ATPases. *Physiological Reviews,* 87, pp. 1011-1046.

Lutsenko, S., Barnes, N.L., Bartee, M.Y. and Dmitriev, O.Y. 2007. Function and regulation of human copper-transporting ATPases. *Physiological Reviews,* 87, pp. 1011-1046.

Osredkar, J. and Sustar, N. 2011. Copper and Zinc, Biological Role and Significance of Copper/Zinc Imbalance. *Journal of Clinical Toxicology,* S3, pp.1-18.

Mackenzie, A.M., Drennan, M., Rowan, T.G., Dixon, J.B. and Carter, S.D. 1997a. Effect of transportation and weaning on humoral immune responses of calves. *Research in Veterinary Science,* 63, pp. 227-230.

Mackenzie, A.M., Illingworth, D.V., Jackson, D.W. and Telfer, S.B. 1997b. The use of caeruloplasmin activities and plasma copper concentrations as an indicator of copper status in ruminants. In: *Proceedings of the 9th International Symposium on Trace Elements in Man and Animals.* Edited by Fischer, P.W.F., L'Abbe, M.R., Cockel, K.A. and Gibson, R.S. Ottawa, Canada.

Mackenzie, A.M., Moeini, M.M. and Telfer S.B. 2001. The effect of a copper, cobalt and selenium bolus on fertility and trace element status of dairy cattle. In: *Fertility in the High-Producing Dairy Cow Occasional Publication*. Edited by Diskin, M.G. British Society of Animal Science, Edinburgh, 26, pp. 423–427.

Maekawa, M., Beauchemin, K.A. and Christensen, D.A. 2002. Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. *Journal of Dairy Science,* 85, pp. 1165-1175.

Manola, D.J., Buehler, P.W., Baek, J.H., Butt, O., Diagnillo, F. and Alayash, A.I. 2008. Acellular haemoglobin attenuates hypoxia-inducible factor-1α (HIF-1α) and its target genes in haemodiluted rats. *Biochemical Journal,* 414, pp. 461-469.

Markossian, K.A. and Kurganov, B.I. 2003. Copper chaperones, intracellular copper trafficking proteins. Function, structure, and mechanism of action. *Biochemistry,* 68, pp. 827-837.

Mason, J., Woods, M. and Poole, D.B.R. 1982. Accumulation of copper and albumin in bovine plasma in *vivo* after intravenous trithiomolybdate administration. *Research in Veterinary Science,* 41, pp 108-113.

Massie, H.R., Ofosu-Appiah, W. and Aiello, V.R. 1993. Elevated serum copper is associated with reduced immune response in aging mice. *Gerontology,* 39, pp. 136-145.

Matsubayashi, H., kitaya, K., Yamaguchi, K., Nishiyama, R., Takaya, Y. and Ishikawa, T. 2017. Is a high serum copper concentration a risk factor for implantation failure? *BMC Research Notes,* 10, pp. 1-6.

Matsuda, I., Pearson, T. and Holtzman, N.A. 1974. Determination of apoceruloplasmin by radioimmunoassay in nutritional copper deficiency, Menke's kinky hair syndrome, Wilson's disease, and umbilical cord blood. *Pediatric Research,* 8. pp. 821-824.

McArt, J.A.A., Nydam, D.V. and Oetzel, G.R. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. *Journal of Dairy Science,* 95, pp. 5056-5066.

McDonald, P., Edwards, R.A., Morgan, C.A., Greenhalgh, J.F.D., Sinclair, L.A. and Wilkinson, R.G. 2011. *Animal nutrition 7th edition.* Prentice Hall, Harlow.

McDowell, L.R. 1985. Copper, molybdenum and sulfur. *Nutrition of Grazing Ruminants in Warm Climates.* Edited by Cunha, T.J. and McDowell, L.R. Academic Press Ltd., New York, pp. 237-255.

McElwee, M.K., Song, M.O. and Freedman, J.H. 2009. Copper activation of NF-kappaB signaling in HepG2 cells. *Journal of Molecular Biology,* 393, pp. 1013-1021.

McFarlane, J.D., Judson, G.J. and Gouzos, J. 1990. Copper deficiency in ruminants in the south east of south australia. *Australian Journal of Experimental Agriculture,* 30, pp. 187-193.

McGarry, J.D. and Brown, N.F. 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *European Journal of Biochemistry,* 244, pp. 1-14.

McLeod, B.J., Haresion, W. and Lamming, G.E. 1982. The induction of ovulation and luteal function in seasonally anoestrus ewes treated with small-dose multiple injections of GnRH. *Journal of Reproduction and Fertility,* 65, pp. 215-221.

McNaught, M.L., Owen, E.C., and Smith, J.B. 1950. The utilization of non-protein nitrogen in the bovine rumen. *Biochemistry Journal,* 46, pp. 36-43.

Mercer, J.F.B. and Llanos, R.M. 2002. Molecular and cellular aspects of copper transport in developing mammals. *Journal of Nutrition,* 133, pp. 1481S-1484S.

Mew, N.A., Simpson, K.L., Gropman, A.L., Lanpher, B.C., Chapman, K.A. and Summar, M. 2017. Urea cycle disorders overview. *GenReviews,* pp. 1-19.

Mills, C.F. and Davis, G.K. 1987. Molybdenum. In: *Trace Elements in Human and Animal Nutrition.* Edited by Mertz, W. Academic Press, New York, 1, pp. 429-457.

Mills, C.F., Dalgarno, A.C., and Wenham, G. 1976. Biochemical and pathological changes in tissues of Friesian cattle during the experimental induction of copper deficiency. *British Journal of Nutrition*, 35, pp. 309-311.

Miltimore, J.E. and Mason, J.L. 1971. Copper to molybdenum ratio and molybdenum and copper concentrations in ruminant feeds. *Canadian Journal of Animal Science,* 51, pp. 193-200.

Minatel, L. and Carfagnini, J.C. 2000. Copper deficiency and immune response in ruminants. *Nutrition Research,* 20, pp. 1519-1529.

Ministry of Agriculture, Fisheries and Food (MAFF). 1986. *The Analysis of Agricultural Materials: A Manual of the Analytical Methods Used by the Agricultural Development and Advisory Service.* 3rd edition. H.M. Stationary Office, UK.

Ministry of Agriculture, Fisheries and Food (MAFF). 1992. *Feed Composition: UK Tables of Feed Composition and Nutritive Value for Ruminants.* 2nd edition. Chalcombe Publications, Nottingham, UK.

Ministry of Health, Labour, and Welfare (MHLW). 2015a. Health and nutrition survey in Japan, 2015.

Ministry of Health, Labour, and Welfare (MHLW). 2015b.Dietary reference intake in Japan, 2015.

Minson, D.J. 1990. *Forage in ruminant nutrition.* Academic Press, San Diego.

Minson, D.J. and McDonald, C.K. 1987. Estimating forage intake from the growth the growth of beef cattle. *Tropical Grassland,* 21, pp. 116-122.

Miranda, M., Cruz, J.M., López-Alonso, M. and Benedito, J.L. 2006, Variations in liver and blood copper concentrations in young beef cattle raised in north-west Spain: associations with breed, sex, age and season. *Animal Science,* 82, pp. 253–258.

Miranda, M., Benedito, J.L., Gutiérrez, B., García-Vaquero, M., Blanco-Penedo, I. and López-Alonso, M. 2010. *Journal of Veterinary Investigation,* 22, pp. 277-281.

Morales, M.S., Palmquist, D.L. and Weiss, W.P. 2000. Effects of fat source and copper on unsaturation of blood and milk triacylglycerol fatty acids in Holstein and Jersey cows. *Journal of Dairy Science,* 83, pp. 2105-2111.

Morales-Roura, J.S., Zarco, L., Hernandez,-Ceron, J., Rodriguez, G. 2001. Effect of short-term treatment with bovine somatropin at estrus on conception rate and luteal function of repeat-breeding dairy cows. *Theriogenology,* 55, pp. 1831-1841.

Morimoto, I., Ninomiya, H., Komatsu, K. and Satho, M. 1986. Pregnancy and penicillamine treatment in a patient with wilson's disease. *Japanese Journal of Medicine*, 25, pp. 59-62.

Misra, H.P. and Fridovich, I. 1977. Superoxide dismutase: a photochemical augmentation assay. *Archives of Biochemistry and Biophysics,* 181, pp. 308-312.

Mulryan, G. and Mason, J. 1987. The inhibition of bovine serum amine oxidase by thiomolybdates *in vivo* and *in vitro*. *Biochemical Society Transactions,* 15, pp. 248-249.

Mulryan, G. and Mason, J. 1992. Assessment of liver copper status in cattle from plasma copper and plasma copper enzymes. *Annales de Recherches Veterinaires 2*, pp. 233-238.

Nagarajan, R.P., Chen, F., li, W., Vig, E., Harrington, M.A., Nakshatri, H. and Chen, Y. 2000. Repression of transforming-growth-factor-β-mediated transcription by nuclear factor κB. *Biochemistry Journal,* 348, pp. 591-596.

National Research Council (NRC). 1980. *Mineral tolerance of domesticated animals.* National Academy Press, Washington D.C.

National Research Council (NRC). 1989. *Nutrient requirements of dairy cattle.* 6th revised edition. National Academy Press, Washington, D.C.

National Research Council (NRC). 2001. *Nutrient requirements of dairy cattle.* 7th revised edition. National Academy Press, Washington D.C.

National Research Council (NRC). 2005. *Mineral tolerance of animals.* Revised edition. National Academies Press, Washington D.C.

Niles, G.A., Morgan, M. and Edwards, W.C. 2002. The relationship between sulfur, thiamine and polioencephalomalacia a review. *Bovine Practice,* 36, pp. 93-99.

Nockels, C.F., DeBonis, J. and Torrent, J. 1993. Stress induction affects copper and zinc balance in calves fed organic and inorganic copper and zinc sources. *Journal of Animal Science,* 71, pp. 2539-2545.

Nyasae, L., Bustos, R., Braiterman, L., Eipper, B. and Hubbard, A. 2007. Dynamics of endogenous ATP7A (menkes protein) in intestinal epithelial cells: Copper-dependent redistribution between two intracellular sites. *American Journal of Physiology-Gastrointestinal and Liver Physiology,* 292, pp. G1181-G1194.

Nydam, D.V., Ospina, P.A., McArt, J.A., Oetzel, G. and Overton, T.R. 2013. Monitoring negative energy balance in transition dairy cows for herd results. In: *Proceedings of the*

22nd Tri-State Dairy Nutrition Conference. Michigan State University, Fort Wayne, Indiana, USA, pp. 9-22.

Oba, M. and Allen, M.S. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: Effects on dry matter intake and milk yield of dairy cows. *Journal of Dairy Science,* 82, pp. 590-596.

O'Dell, B.L. 1976. Biochemistry of Copper. *Medicine Clinical of North America,* 60, pp. 687-703.

Ogra, T., Ohmichi, M. and Suzuki, K.T. 1996. Mechanisms of selective copper removal by tetrathiomolybdate from metallothionein in LEC rats. *Toxicology,* 106, pp. 75-83.

Ospina, P.A., Nydam, D.V., Stokol, T. and Overton, T.A. 2010. Evaluation of nonesterified fatty acids and β-hydroxybutarate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. *Journal of Dairy Science,* 93, pp. 546-554.

Oster, G. and Salgo, M.P. 1975. The copper intrauterine device and it's mode of action. *New England Journal of Medicicne,* 293, pp. 432-438.

Packer, E.L., Clayton, E.H., Cusack, P.M.V. 2011. Rumen fermentation and liveweight gain in beef cattle treated monensin and grazing lush forage. *Australian Veterinary Journal,* 89, pp. 338-345.

Parliament, U.K. 2012. Animals (Scientific Procedures) Act 1986. Amendment Regulations 2012. The Stationary Office, Norwich, UK.

Paula, J.P.L., Leal, P.V., Pupin, R.C., Lima, S.C., Souza, M.A.S., Santos, A.A., Lemos, R.A.A. and Gomes, D.C. 2018. Healing of brain lesions in sheep recovered from amprolium-induced polioencephalomalacia. *Pesquisa Veterinária Brasileira,* 38, pp. 806-810.

Paulina, J. and Tadeusz, S. 2011. Acute phase proteins in cattle. In: *Acute Phase Proteins as Early Non-Specific Biomarkers of Human and Veterinary Diseases.* Edited by Francisco Veas. IntechOpen Ltd, London, UK.

Paynter, D.I. The diagnosis of copper insufficiency. In: *Copper in Animals and Man.* Edited by McHowell, J. and Gawthorne, J.M. CRC Press, Florida, USA, 101, pp. 101-119.

Percival, S.S. 1988. Copper and immunity. *American Journal of Clinical Nutrition,* 67, pp. 1064S-1068S.

Perrin, D.J., Schiefer, H.B. and Blakley, B.R. 1990. Chronic copper toxicity in a dairy herd. *Canadian Veterinary Journal,* 31, pp. 629-632.

Phillippo, M. and Graca, D.S. 1983. Biliary copper secretion in cattle. In: *Proceedings of the nutrition society,* pp. 46A.

Phillippo, M., Humphries, W.R., Lawrence, C.B. and Price, J. 1982. Investigation of the effect of copper therapy on fertility in beef suckler herds. *Journal of Agricultural Science,* 99, pp.359-364.

Phillippo, M., Humphries, W.R., and Garthwaite, P.H. 1987a. The effect of dietary molybdenum and iron on copper status and growth in cattle. *The Journal of Agricultural Science,* 109, pp. 315-320.

Phillippo, M., Humphries, W.R., Atkinson, T., Henderson, G.D. and Garthwaite, P.H. 1987b. The effect of dietary molybdenum and iron on copper status, puberty, fertility and oestrus cycles in cattle. *The Journal of Agricultural Science,* 109, pp. 321-336.

Plaizier, J.C., Krause, D.O., Gozho, G.N. and McBride, B.W. 2008. Subacute ruminal acidosis in dairy cows: The physiological causes, incidence and consequences. *Veterinary Journal,* 176, pp. 21-31.

Pollock, J.M., Rowan, T.G., Dixon, J.B., Carter, S.D. and Kelly, D.F. 1991. Estimation of immunity in the developing calf: Cellular and humoral responses to keyhole limpet haemocyanin. *Veterinary Immunology and Immunopathology,* 29, pp. 105-217.

Price, J., Will, A.M., Paschaleris, G. and Chesters, J.K. 1987. Identification of thiomolybdates in digesta and plasma from sheep after administration of ^{99}Mo-labelled compounds in the rumen. *British Journal of Nutrition,* 58, pp. 127-138.

Prohaska, J.R. 2006. Copper. In: *Present Knowledge in Nutrition 7th edition.* Edited by Filer, L.J. and Ziegler, E.E. International Life Science Institute-Nutrition Foundation, Washington D.C.

Prohaska, J.R. 2012. Copper. In: *Present Knowledge in Nutrition 10th edition.* Edited by Erdman, J.W., Macdonald, I.A. and Zeisel, S.H. International Life Science Institute-Nutrition Foundation, Washington D.C.

Prohaska, J.R., Gybina, A.A., Broderius, M. and Brokate, B. 2005. Peptidylglycine-α-amidating monooxygenase activity and protein are lower copper-deficient rats and suckling copper-deficient mice. *Archives of Biochemistry and Biophysics,* 434, pp. 212-220.

Pruszkowski, E. and Neubauer, K. 2017. *The NexION 2000: A perfect tool for the determination of trace elements in blood and serum.* PerkinElmer, Waltham, USA.

Puls, R. 1994. Copper. In: *Mineral levels in animal health.* 2nd edition. Sherpa International, Clearbook, Canada, pp. 83-88.

Richter, E.L., Drewnoski, M.E. and Hansen, S.L. 2012. Effects of increased dietary sulfur on beef mineral status, performance, and meat fatty acid composition. *Journal of Animal Science,* 90, pp. 3945-3953.

Rivera, H., Lopez, H. and Fricke, P.M. 2004. Fertility of Holstein dairy heifers after synchronization of ovulation and timed AI or AI after removed tail chalk. *Journal of Dairy Science,* 87, pp. 2051-2061.

Reynolds, C.K., Sutton, J.U.D. and Beever, D.E. 1997. Effects of feeding starch to dairy cattle on nutrient availability and production. In: *Recent Advances in Animal Nutrition.* Edited by Garnsworthy, P.C. and Wiseman, J. Nottingham University Press, Nottingham, UK.

Roche, J.R. 2007. Milk production responses to pre- and postcalving dry matter intake in grazing dairy cows. *Livestock Science,* 110, pp. 12-24.

Roche, J.R., Berry, D.P., Lee, J.M., Macdonald, K.A. and Boston, R.C. 2007. Describing the body condition score change between successive calvings: A novel strategy generalizable to diverse cohorts. *Journal of Dairy Science,* 90, pp. 4378-4396.

Roche, J.R., Friggens, N.C., Kay, J.K, Fisher, M.W., Stafford, K.J. and Berry, D.P. 2009. Invited Review: Body condition score and its association with dairy cow productivity, health and welfare. *Journal of Dairy Science,* 92, pp. 5769-5801.

Rodney, R.M., Celi, P., Scott, W., Breinhild, K. and Lean, I.J. 2015. Effects of dietary fat on fertility of dairy cattle: A meta-analysis and meta-regression. Journal of Dairy Science, 98, pp. 5601-5620.

Rodríguez-Lecompte, J.C., Kroeker, A.D., Ceballos-Márquez, A., Li, S., Plaizier, J.C. and Gomez, D.E. 2014. Evaluation of the systemic innate immune response and metabolic

alterations of nonlactating cows with diet-induced subacute ruminal acidosis. *Journal of Dairy Science,* 97, pp. 7777-7787.

Roland, L., Drillich, M. and Iwersen, M. 2014. Hematology as a diagnostic tool in bovine medicine. *Journal of Veterinary Diagnostic Investigation,* 26, pp. 592-598.

Rook, A.H., Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Sporn, M.B., Burlington, D.B., Lane, H.C. and Fauci, A.S. 1986. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. *Journal of Immunology,* 136, pp. 3916-3920.

Rosendo, O. and McDowell, L.R. 2003. Lipid dry matter and lipids in periparturient dairy cows. *Acta Veterinaria Brno,* 72, pp. 541-546.

Roughead, Z.K. and Lukaski, H.C. 2003. Inadequate copper intake reduces serum insulin-like growth factor-1 and bone strength in growing rats fed graded amounts of copper and zinc. *Journal of Nutrition,* 133, pp. 442-448.

Rucker, R., Romero-Chapman, N., Wong, T., Lee, J., Steinberg, F., McGee, C., Clegg, M., Reiser, K., Kosonen, T., Urie-Hare, J., Murphy, J. and Keen, C. 1996. Modulation of lysyl oxidase by dietary copper in rats. *Journal of Nutrition,* 126, pp. 51-60.

Rucker, R., Kosonen, T., Clegg, M.S., Mitchel, A.E., Rucker, B.R., Urie-Hare, J.Y., and Keen, C.L. 1998. Copper, lysyl oxidase and extracellular matrix protein cross-linking. *American Journal of Clinical Nutrition,* 67, pp. 996s-1002s.

Saenko, E.L., Yaropolov, A.I., and Harris, E.D. 1994. Biological functions of ceruloplasmin expressed through copper-binding sites and a cellular receptor. *The Journal of Trace Elements in Experimental Medicine,* 7, pp. 69-88.

Saker, K.E., Eversole, D.E. and Williams, E.I. 1994. Effect of copper supplementation and vaccination on cellular immune response in growing beef calves. *Journal of Animal Science,* 72 (Supplement 1), pp. 131 (Abstract).

Sass-Kortsak, A. and Beam, A.G. 1978. Hereditary disorders of copper metabolism. In: *The Metabolic Basis of Inherited Disease 4th edition*. Edited by Stanbury, J.B., Wyngaarden, J.B. and Fredrickson, D.S. McGraw-Hill Book Company, New York, pp. 1098-1260.

Sattar, N., Scott, H.R., McMillan, D., Talwar, D., O'Reilly, D.S. and Fell, G.S. 1997. Acute-phase reactants and plasma trace element concentrations in non-small cell lung cancer patients and controls. *Nutrition and Cancer,* 28, pp. 308-312.

Sauvant, D., Perez, J.M. and Tran, G. 2002. *Tables of composition and nutritional value of feed materials 2nd edition,* Wageningen Academic Publishers, Netherlands.

Saylor, W.W., Morrow, F.D. and Leach, R.M., Jr. 1980. Copper-and zinc-binding proteins in sheep liver and intestine: effects of dietary levels of the metals. *The Journal of Nutrition,* 110, pp. 460-468.

Scaletti, R.W., Trammell, D.S., Smith, B.A. and Harmon, R.J. 2003. Role of dietary copper in enhancing resistance to escherichia coli mastitis. *Journal of Dairy Science,* 86, pp. 1240-1249.

Schillo, K.K., Hall, J.B. and Hileman, S.M. 1992. Effects of nutrition and season on the onset of puberty in the beef heifer. *Journal of Animal Science,* 70, pp. 3994-4005.

Schoonmaker, J.P. and Beitz, D.C. 2012. Hydrogen sulphide: Synthesis, physiological roles and pathology associated with feeding cattle maize co-products of the ethanol industry. In: *Biofuel co-products as livestock feed: Opportunities and challenges. Volume 1.* Edited by Makkar, H.P.S. Food and Agriculture Organization of the United Nations, Rome, Italy, pp.101-114.

Schwarz, F.J. and Kirchgessner, M. 1978. Copper and zinc contents in milk and plasma of cows after high nutritional copper supplements. *Zeitschrift für Lebensmitteluntersuchung und -Forschung,* 166, pp. 5-8.

Sefdeen, S.M. 2017. *Effects of iron on copper metabolism in sheep.* Ph.D. Thesis, Harper Adams University.

Shim, H., Harris, Z.L., King, J.C., Allen, L.H., Coughlin, J.R., Hambidge, K.M., Keen, C.L., Lönnerdal, B.L. and Rucker, R.B. 2003. Genetic defects in copper metabolism. *Journal of Nutrition,* 133, pp. 1527S-1531S.

Sejrsen, K. 1994. Relationships between nutrition, puberty and mammary development in cattle. *Proceedings of the Nutrition Society,* 53, 103-111.

Sejrsen, K., Huber, J.T., and Tucker, H.A. 1983. Influence of amount fed on hormone concentrations and their relationship to mammary growth in heifers. *Journal of Dairy Science,* 66, pp. 845-855.

Sevinc, M., Basoglu, A., Birdane, F.M. and Boydak, M. 2001. Liver function in dairy cows with fatty liver. *Revvue de médicine vétérinare,* 152, pp. 297-300.

Slot, J.W., Geuze, H.J., Freeman, B.A., and Crapo, J.D. 1986. Intracellular localization of the copper-zinc and manganese superoxide dismutases in rat liver parenchymal cells. *Laboratory Investigations,* 55, pp. 363-371.

Sinclair, L.A. and Atkins, N.E. 2015. Intake of selected minerals on commercial dairy herds in central and northern England in comparison with requirements. *Journal of Agricultural Science,* 153, pp. 743-752.

Sinclair, L.A. and Mackenzie, A.M. 2013. Mineral nutrition of dairy cows: supply vs requirements. In: *Recent Advances in Animal Nutrition.* Edited by Garnsworthy, P.C. and Wiseman, J. Context Products Ltd, Leicestershire, UK, pp. 13-30.

Sinclair, L.A, Bond, A.J., Huntington, J.A. and Readman, R.J. 2007. Effect of rate of substitution of processed, urea-treated whole-crop wheat for grass silage on the intake, milk production and diet digestibility in dairy cows and ruminal metabolism in vitro. *Animal,* 1, pp. 601-611.

Sinclair, L.A., Hart, K.J., Johnson, D. and Mackenzie, A.M. 2013. Effect of inorganic or organic copper fed without or with added sulfur and molybdenum on the performance, indicators of copper status, and hepatic mRNA in dairy cows. *Journal of Dairy Science,* 96, pp. 4355-4367.

Sinclair, L.A., Johnson, D., Wilson, S. and Mackenzie, A.M. 2017. Added dietary sulfur and molybdenum has a greater influence on hepatic copper concentration, intake, and performance in Holstein-Friesian dairy cows offered a grass silage-rather than corn silage-based diet. *Journal of Dairy Science,* 100 pp. 1-12.

Singh, I. 1997. Biochemistry of peroxisomes in health and disease. *Molecular Cell Biochemistry,* 167, pp. 1-29.

Smith, B.S.W., Field, A.C. and Suttle, N.F. 1968. Effect of intake of copper, molybdenum and sulphate on copper metabolism in the sheep. *Journal of Comparative Pathology,* 78, pp. 449-461.

Sorners, G.F. 1983. The affinity of onion cell walls for calcium ions. *American Journal of Botany,* 60, pp. 987.

Spais, A.G., Lazaridis, T.K., and Agiannidis, A.K. 1968. Studies on Sulphur metabolism in sheep in association with copper deficiency. *Research in Veterinary Science,* 9, pp. 337-344.

Spears, J.W., Lloyd, K.E. and Fry, S.R. 2011. Tolerance of cattle to increased sulfur and effect of dietary cation-anion balance. *Journal of Animal Science,* 89, pp. 2502-2509.

Spellberg, B. and Edwards, J. 2001 Type 1/ Type 2 immunity in infectious diseases. *Clinical Infectious Disease,* 32, pp. 76-102.

Standish, J.F., Ammerman, C.B., Simpson, C.F., Neal, F.C. and Palmer, A.Z. 1969. Influence of graded levels of dietary iron, as ferrous sulphate, on performance and tissue mineral composition of steers. *Journal of Animal Science,* 29, pp. 496-503.

Starbuck, M.J., Dailey, R.A. and Inskeep, E.K. 2004. Factors affecting retention of early pregnancy in dairy cattle. *Animal Reproductive Science,* 84, pp. 27-39.

Starbuck, M.J., Inskeep, E.K. and Dailey, R.A. 2006. Effect of a single growth hormone (rbST) treatment at breeding on conception rates and pregnancy retention in dairy and beef cattle. *Animal Reproduction Science,* 93, pp. 349-359.

Stearman, R., Yuan, D.S., Yamaguchi-Iwa, Y., Klausner, R.D., and Dancis, A. 1996. A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science,* 271, pp. 1552-1557.

Steele, M.A., Croom, J.C., Kahler, M., AlZahal, O., Hook, S.E., Plaizier, K. and McBride, B.W. 2011. Bovine rumen epithelium undergoes rapid structural adaptations during grain-induced subacute ruminal acidosis. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology,* 300, pp. 1515-1523.

Stipanuk, M.H. 2000. *Biochemical and physiological aspects of human nutrition,* Saunders, W.B., Pennsylvania, USA.

Stockdale, C.R. 2001. Body condition at calving and the performance of dairy cows in early lactation under Australian conditions: A review. *Australian Journal of Experimental Agriculture,* 41, pp. 1-9.

Storry, J.E. 1961. Studies on calcium and magnesium in the alimentary tract of sheep. 2. The effect of reducing the acidity of abomasal digesta in vitro on the distribution of calcium and magnesium. *Journal of Agricultural Science,* 57, pp. 103.

Stoszek, M.J., Mika, P.G., Oldfield, J.E. and Weswig, P.H. 1986. Influence of copper supplementation on blood and liver copper in cattle fed tall fesue or quackgrass. *Journal of Animal Science,* 62, pp. 263-271.

Strickland, J.M., Lyman, D., Sordillo, L.M., Herdt, T.H. and Buchweitz, J.P. 2019. Effects of super nutritional hepatic copper and accumulation on hepatocyte health and oxidative stress in dairy cows. *Veterinary Medicine International*, 29, pp. 1-9.

Sumner, J.M. and McNamara, J.P. 2007. Expression of lipolytic genes in the adipose tissue of pregnant and lactating Holstein dairy cattle. *Journal of Dairy Science,* 90, pp. 5237-5246.

Suttle, N.F. 1974. Recent studies on the copper-molybdenum-sulphur inter-relationship. *Proceedings of the Nutritional Society,* 33, pp. 299-305.

Suttle, N.F. 1975. Changes in the availability of dietary copper to young lambs associated with age and weaning. *Journal of Agricultural Science,* 84, pp. 255-261.

Suttle, N.F. 1983. Effects of molybdenum concentration in fresh herbage, hay and semi-purified diets on the copper metabolism of sheep. *Journal of Agricultural Science,* 100, pp. 651-656.

Suttle, N.F. 1986. Copper deficiency in ruminants; recent developments. *Veterinary Record,* 119, pp. 519-522.

Suttle, N.F. 1987. The nutritional requirement for copper in animals and man. In: *Copper in Animals and Man. Volume 1.* Edited by Howell, J.M. and Gawthorne, J.M. CRC Press, Florida, pp. 21-44.

Suttle, N.F. 1991. The interactions between copper, molybdenum, and sulphur in ruminant nutrition. *Annual Review of Nutrition,* 11, pp. 121- 140.

Suttle, N.F. 1994. Meeting the copper requirements of ruminants. In: *Recent Advances in Animal Nutrition.* Edited by Garnsworthy, P.C. and Cole, D.J.A. Nottingham University Press, Nottingham, UK, pp. 173-188.

Suttle, N.F. 2002. Copper deficiency- how has the disease and its diagnosis in the last 15 years? *Cattle Practice,* 10, pp. 275-278.

Suttle, N.F. 2008. Relationships between the concentrations of trichloroacetic acid-insoluble copper and caeruloplasmin in the serum of cattle from areas with different soil concentrations of molybdenum. *Veterinary Record,* 162, pp. 237-240.

Suttle, N.F. 2010. *Mineral nutrition of livestock 4th edition*, CABI Publishing, Wallingford, UK.

Suttle, N.F. 2016. Reducing the risk of copper toxicity in dairy cattle. *Veterinary Record,* 178, pp. 196-196.

Suttle, N.F. and McLauchlin, M. 1976. Predicting the effects of dietary molybdenum and sulphur on the availability of copper to ruminants. In: *Proceedings of the Nutritional Society,* 35, pp. 22A.

Suttle, N.F. and Field, A.C. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. *Journal of Comparative Pathology,* 93, pp. 379-389.

Suttle, N.F. and McMurray, C.H. 1983. Effects of dietary supplements of thiomolybdates on copper and molybdenum metabolism in sheep. *Journal of Comparative Pathology,* 93, pp. 379-389.

Suttle, N.F. and Peters, D.W. 1985. Rumen sulphide metabolism as a major determinant of the availability of copper to ruminants. In: *Proceedings of the Fifth International Symposium on Trace Elements in Man and Animals.* Edited by Mills, C.F., Bremner, I. and Chesters, J.K. Farnham Royal, Aberdeen, UK.

Suttle, N.F., Field, A.C., and Barlow, R.M. 1970. Experimental copper deficiency in sheep. *Journal of Comparative Pathology,* 82, pp. 151-162.

Suttle, N.F., Jones, D.G., Woolliams, C. and Woolliams, J.A. 1987. Heinz body aneamia in lambs with deficiencies of copper or selenium. *British Journal of Nutrition,* 58, pp. 539-548.

Suttle, N.F., Knox, D.P., Angus, K.W., Jackson, F. and Coop, R. 1990. Dietary molybdenum may enhance the inflammatory reaction to and hence rejection of gut nematodes in lambs. In: *Proceedings of the Nutritional Society,* 48, pp. 71A.

Suttle, N.F., Brebner, J. and Pass, R. 1996. A comparison of the availability of copper in four whisky distillery by-products with that in copper sulfate for lambs. *Animal Science,* 62, pp. 689-690.

Suttle, N.F., Lewis, R.M. and Small, J.N.W. 2002. Effects of breed and family on rate of copper accretion in the liver of purebred charollais, suffolk and texel lambs. *Animal Science,* 75, pp. 295-302.

Sutton, J.D. 1989. Altering milk composition by feeding. *Journal of Dairy Science,* 72, pp. 2801-2814.

Swanson, G. 1991. Individual animal models. *Holstein Friesian Journal,* 73, pp. 625-626.

Tafaj, M., Zebeli, Q., Baes, C., Steingass, H. and Drochner, W. 2007. A meta-analysis examining effects of particle size of total mixed rations on intake, rumen digestion and milk production in high-yielding dairy cows in early lactation. *Animal Feed Science and Technology,* 138, pp. 137-161.

Tapiero, H., Townsend, D.M. and Tew, K.D. 2003. Review: Trace elements in human physiology and pathology. Copper. *Biomedicine and Pharmacotherapy,* 57, pp. 386-398.

Tayyab, U., Wilkinson, R.G., Charlton, G.L., Reynolds, C.K. and Sinclair, L.A. 2018. Grass silage particle size when fed with or without maize silage alters performance, reticular pH and metabolism of Holstein-Friesian dairy cows. *Animal,* 13, pp. 524-532.

Telfer, S.B., Kendall, N.R., Illingworth, D.V. and Mackenzie, A.M. 2004. Molybdenum toxicity in cattle: an underestimated problem. *Cattle practice,* 12, 259-263.

Terada, K., Kawarda, Y., Miura, N., Yasui, O., Koyama, K. and Sugiyama, T. 1995. Copper incorporation into caeruloplasmin in rat livers. *Biochemica et Biophysica Acta,* 1270, pp. 58-62.

Thomas, T.A. 1977. An automated procedure for the determination of soluble carbohydrates in herbage. *Journal of Agricultural Science,* 28, pp. 639-642.

Thomas, C. 2004. *Feed Into Milk.* Nottingham University Press, Nottingham, UK.

Thompson, L.H., Wise, M.B., Harvey, R.W., and Barrick, E.R. 1972. Starea, urea and sulfur in beef cattle rations. *Journal of Animal Science,* 35, pp. 474-480.

Todd, J.R. 1969. Chronic copper toxicity of ruminants. *Nutrition Society Proceedings,* 28, pp. 189-198.

Torre, P.M., Harmon, R.J., Hemken, R.W., Clark, T.W., Trammell, D.S. and Smith, B.A. 1996. Mild dietary copper insufficiency depresses blood neutrophil function in dairy cattle. *Journal of Nutritional Immunology (USA),* 4, pp. 3-24.

Tough, D.F. and Sprent, J. 1995. Lifespan of lymphocytes. *Immunology Research,* 14, pp. 1-12.

Truong, D.H., Eghbal, M.A., Hindmarsh, W., Roth, S.H. and O'Brien, P.J. 2006. Molecular mechanisms of hydrogen sulfide toxicity. *Drug Metabolism and Disposition,* 38, pp. 733-774.

Turner, J.C., Shanks, V., Osborn, P.J. and Gower, S.M. 1987. Copper absorption in sheep. *Comparative Biochemistry and Physiology*, 86, pp. 147-296.

Turnland, J.R., Jacob, R.A., Keen, C.L., Strain, J.J., Kelley, D.S., Domek, J.M., Keyes, W. R., Ensunsa, J.L., Lykkesfeldt, J. and Coulter, J. 2004. Long-term high copper intake: Effects on indexes of copper status, antioxidant status, and immune function in young men. *American Journal of Clinical Nutrition,* 79, pp. 1037-1044.

Tyrrell, H.F. and Reid, J.T. 1965. Prediction of the energy value of cow's milk. *Journal of Dairy Science,* 48, pp. 1215-1223.

Uchida, E., Katoh, N. and Takahashi, K. 1993. Appearance of haptoglobin in serum from cows at parturition. *Journal of Veterinary Medical Science,* 55, pp. 893-894.

Underwood, E.J. 1977. Copper. In: *Trace Elements in Human and Animal Nutrition.* 4th edition. Edited by Underwood, E.J. Academic Press, New York, pp. 56-108.

Underwood, E.J. 1981. *The Mineral Nutrition of Livestock.* 2nd edition, CABI Publishing, London, UK.

Underwood, E.J. and Suttle, N.F. 1999. *The mineral nutrition of livestock.* 3rd edition, CABI Publishing, New York, USA.

Uwituze, S., Parsons, G.L., Karges, K.K., Gibson, M.L., Hollis, L.C., Higgins, J.J., and Drouillard, J.S. 2011. Effects of distillers grains with high sulfur concentration on ruminal

fermentation and digestibility of finishing diets. *Journal of Animal Science,* 89, pp. 2817-2828.

Valko, M., Morris, H. and Cronin, M.T.D. 2005. Metals, toxicity and oxidative stress. *Current Medicinal Chemistry,* 12, pp. 1161-1208.

Vanholder, T., Papen, J., Bemers, R., Vertenten, G. and Berge, A.C. 2015. Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. *Journal of Dairy Science,* 98, pp. 880-888.

Vierboom, M.M., Engle, T.E. and Kimberling, C.V. 2002. Effects of gestational status, on apparent absorption and retention of copper and zinc in mature angus cows and suffolk ewes. *Asian-Australian Journal of Animal Sciences,* 16, pp. 515-518.

Van der Vliet, A. 2015. Antioxidant defenses in the lung. In: *Comparative Biology of the normal lung. 2nd edition.* Edited by Parent, R.A. Elsevier Inc, Netherlands, pp. 489-507.

Van Erdenburg, F.J.C.M., Loeffler, H.S.H and Van Vliet, J.H. 1996. Detection of estrus in dairy cows: A new approach to an old problem. *Veterinary Quarterly,* 18, pp. 52-54.

Van Erdenburg, F.J.C.M., Karthaus, D., Taverne, M.A.M., Merics, I. and Szenci, O. 2002. The relationship between estrus behavioral score and time of ovulation in dairy cattle. *Journal of Dairy Science,* 85, pp. 1150-1156.

Van Soest, P.J., Robertson, J.B. and Lewis. B.A. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, 74, pp. 3583–97.

Vonk, W.I.M., Wijmenga, C., and van de Sluis, B. 2008. Relevance of animal models for understanding mammalian copper homeostasis. *American Journal of Clinical Nutrition,* 88, 840s-845s.

Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. 1993. Isolation of a cadidate gene for menkes disease and evidence that it encodes a copper-transporting ATPase. *Nature Genetics,* 3, pp. 7-13.

Waltner, S.S., McNamara, J.P. and Hillers, J.K. 1993. Relationships of body condition score to production variables in high producing Holstein dairy cows. *Journal of Dairy Science,* 76, pp. 3410-3419.

Wankhade, P.R., Manimaran, A., Kumaresan, A., Jeyakumar, S., Ramesha, K.P., Sejian, V., Rajendran, D. and Varghese, M.R. 2017. Metabolic and immunological changes in transition dairy cows: A review. *Veterinary World,* 10, pp. 1367-1377.

Wang, T. and Guo, Z.J. 2006. Copper in medicine: Homeostasis, chelation therapy and antitumor drug design. *Current Medicinal Chemistry,* 13, pp. 525-537.

Wang, J., Zhu, X., Xie, G., Zhang, Y., Liu, Z., Zhang, L., Zhao, C., Yang, W., Shi, X., Wang, Z. and Liu, G. 2012. Effect of copper on the expression of IGF-1 from chondrocytes in newborn piglets in vitro. *Biological Trace Element Research,* 148, pp. 178-181.

Wany, Z.Y., Poole, D.B.R and Mason, J. 1987. The uptake and intracellular distribution of (^{35}S) trithiomolybdate in bovine liver *in vivo*. *Journal of Inorganic Biochemistry,* 31, pp. 85-93.

Ward, J.D. and Spears, J.W. 1997. Long-term effects of consumption of low-copper diets with or without supplemental molybdenum on copper status, performance, and carcass characteristics of cattle. *Journal of Animal Science (USA),* 75, pp. 3057-3065.

Ward, J.D. and Spears, J.W. 1999. The effects of low-cooper diets with or without supplemental molybdenum on specific immune responses of stressed cattle. *Journal of Animal Science,* 77, pp. 230-237.

Ward, J.D., Spears, J.W. and Kegley, E.B. 1993. Effect of copper level and source (copper lysine vs copper sulfate) on copper status, performance, and immune response in growing steers fed diets with or without supplemental molybdenum and sulfur. *Journal of Animal Science,* 71, pp. 2748-2755.

Ward, J.D., Spears, J.W. and Gengelbach, G.P. 1995. Differences in copper status and copper metabolism among angus, simmental, and charolais cattle. *Journal of Animal Science,* 73, pp. 571-577.

Wakatsuki, T. 1995. Metal oxidoreduction by microbial cells. *Journal of Industrial Microbiology (United Kingdom),* 14, pp. 169-177.

Walshe, J.M. and Cumings, J.N. 1961. *Wilson's disease: Some current concepts.* Blackwell Scientific Publications, Oxford.

Weber, K.M., Boston, R.C. and Leaver, D.D. 1980. A kinetic model of copper metabolism in sheep. *Australian Journal of Agricultural Research* 31: 773-790.

Weber, K.M., Boston, R.C and Leaver, D.D. 1983. The effect of molybdenum and sulfur on the kinetics of copper metabolism in sheep. *Australian Journal of Agricultural Research,* 34, 295-306.

Wee, N.K.Y., Weinstein, D.C., Fraser, S.T. and Assinder, S.J. 2013. The mammalian copper transporters CTR1 and CTR2 and their roles in development and disease. *International Journal of Biochemistry and Cell Biology,* 45, pp. 960-963.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G. and Pollock, J.M. 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology,* 114, pp. 101-111.

Westwood, C.T., Lean, I.J. and Garvin, J.K. 2002. Factors Influencing Fertility of Holstein Dairy Cows: A Multivariate Description. *Journal of Dairy Science,* 85, pp. 3225-3237.

White, H.M. 2015. The role of TCA cycle anaplerosis in ketosis and fatty liver in periparturient dairy cows. *Animals,* 5, pp. 793-802.

Williams, C.L. 2004. The effects of molybdenum, iron and sulphur on copper metabolism and physiology of sheep. Ph.D. Thesis, Harper Adams University.

Wittenberg, K.M. and Devlin, T.J. 1987. Effects of dietary molybdenum on productivity and metabolic parameters of lactating beef cows and their offspring. *Canadian Journal of Animal Science,* 67, pp. 1055-1066.

Woods, M. and Mason, J. 1987. Spectral and kinetic studies on the binding of Trithiomolybdate to bovine and canine serum albumin *in vitro*: The interaction with copper. *Journal of Inorganic Biochemistry,* 30, pp. 261-272.

Wrzesinski, S.H., Wan, Y.Y. and Flavell, R.A. 2007. Transforming growth factor-beta and the immune response: implications for anticancer therapy. *Clinical Cancer Research,* 13, pp. 5262-5270.

Xin, Z., Waterman, D.F., Hemken, R.W. and Harmon, R.J. 1991. Effects of copper status on neutrophil function, superoxide dismutase, and copper distribution in steers. *Journal of Dairy Science,* 74, pp. 3078-3085.

Xu, X., Pin, S., Gathinji, M., Fuchs, R. and Harris, Z.L. 2004. Aceruloplasminemia: an inherited neurodegenerative with impairment of iron homeostasis. *The New York Academy of Sciences,* 1012, pp. 299-305.

Yang, H., Liu, C.N., Wolf, R.M., Ralle, M., Dev, S., Pierson, H., Askin, F., Steele, K.E., Magnuson, T.H., Schweitzer, M.A., Wong, G.W. and Lutsenko, S. 2019. Obesity is associated with copper elevation in serum and tissues. *Metallomics,* 11, pp. 1363-1371.

Yost, G.P., Arthington, J.D., McDowell, L.R., Martin, F.G., Wilkinson, N.S. and Swenson, C.K. 2002. Effect of copper source and level on the rate and extent of copper repletion in Holstein heifers. *Journal of Dairy Science,* 85, pp. 3297-3303.

Young, R.S. 1979. *Cobalt in biology and biochemistry.* Academic Press, London, UK.

Zebeli, Q., Dijkstra, J., Tafaj, M., Steingass, H., Ametaj, B.N. and Drochner, W. 2008. Modeling the adequacy of dietary fiber in dairy cows based on the responses of ruminal pH and milk fat production to composition of the diet. *Journal of Dairy Science,* 91, pp. 2046-2066.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372, pp. 425-432.