Trace elements in zebu cattle: status and impact.
The case of the Gilgel Gibe catchment

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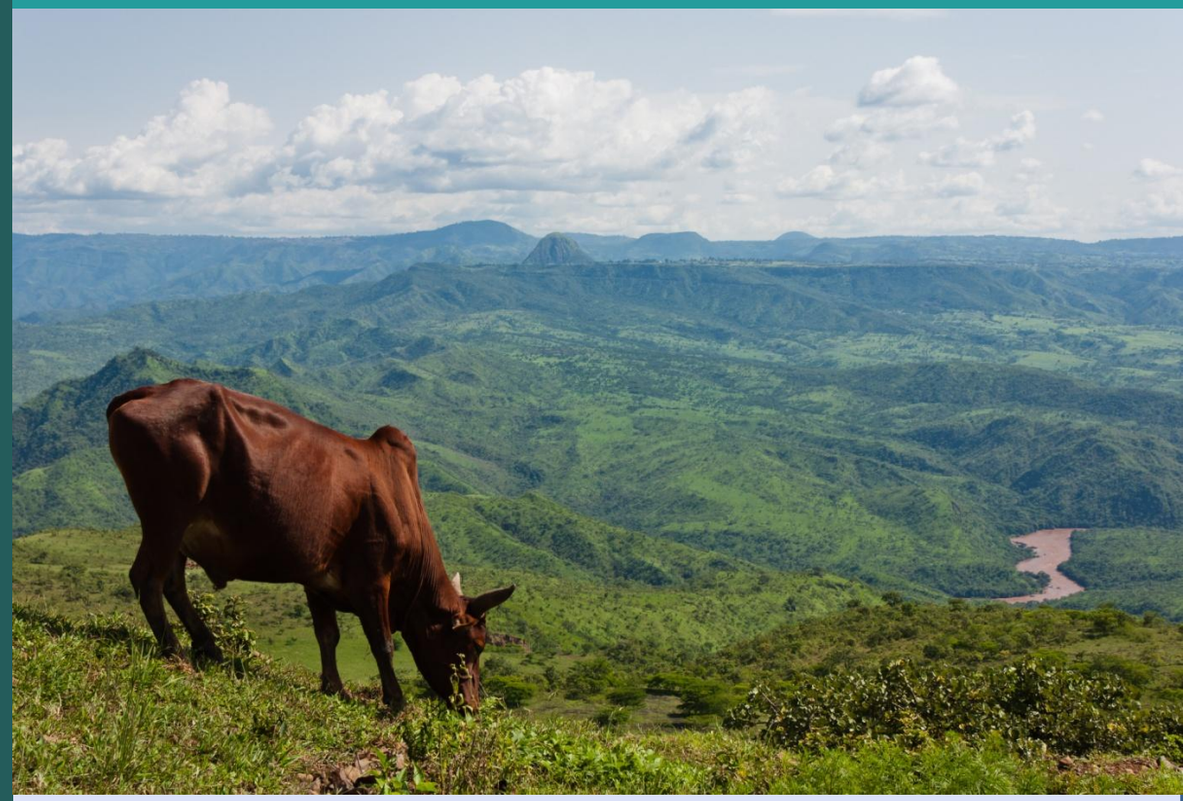

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Trace elements in zebu cattle: status and impact

The case of the Gilgel Gibe catchment, Ethiopia

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences

2013

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“Dər biyabər anbeussa yassər”

Spider webs joined together can catch a lion.

- Ethiopian proverb -

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List of Abbreviations

ADF = acid detergent fibre
ADG = average daily gain
ADL = acid detergent lignin
ALP = alkaline phosphatase activity
asl = above sea level
AST = aspartate aminotransferase activity
BCS = body condition score
B. = *Bos*
BW = body weight
CK = creatine kinase activity
CMT = California Mastitis Test
CP = crude protein
Cp = ceruloplasmin
DM = dry matter
DMI = dry matter intake
DW = dry weight
EE = ether extract
ETD = estimated total dietary
FM = fresh matter
FRAP = ferric reducing ability of plasma
FW = fresh weight
G:F ratio = gain:feed ratio
GGT = γ-glutamyl transferase activity
GM = geometric mean
GSEM = geometric standard error of the mean
GSH-Px = glutathione peroxidase
ICP-MS = inductively coupled plasma mass spectrometry
ICP-OES = inductively coupled plasma optical emission spectrometry
LDH = lactic dehydrogenase activity
MTL = maximum tolerable levels
NAF = Nitisol-Acrisol-Ferralsol association
NAT = neutralizing antibody titer
NDF = neutral detergent fibre
PV = Planosol-Vertisol association
SD = standard deviation
SE = standard error
SEM = standard error of the mean
SOD = superoxide dismutase
TBARS = thiobarbituric acid reactive species

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Introduction

1. Trace elements in cattle

1.1. What's in a name? On metals, minerals and trace elements

Metals are "elements which conduct electricity, have a metallic luster, are malleable and ductile, form cations, and have basic oxides" (Duffus, 2002), whereas the main definition of a *mineral* is stated as follows: "an element or chemical compound that is normally crystalline and that has been formed as a result of geological processes" (Nickel, 1995). A metal can, therefore, be a mineral, and a mineral can be a metal, but not necessarily. For instance, Cu is a metal and a mineral, whereas Se and S are minerals but not metals.

In the context of nutrition, along with vitamins, minerals form the group of micronutrients, substances which are required in very small amounts, yet are also essential for optimal health (Bender, 2007) (Figure 1).

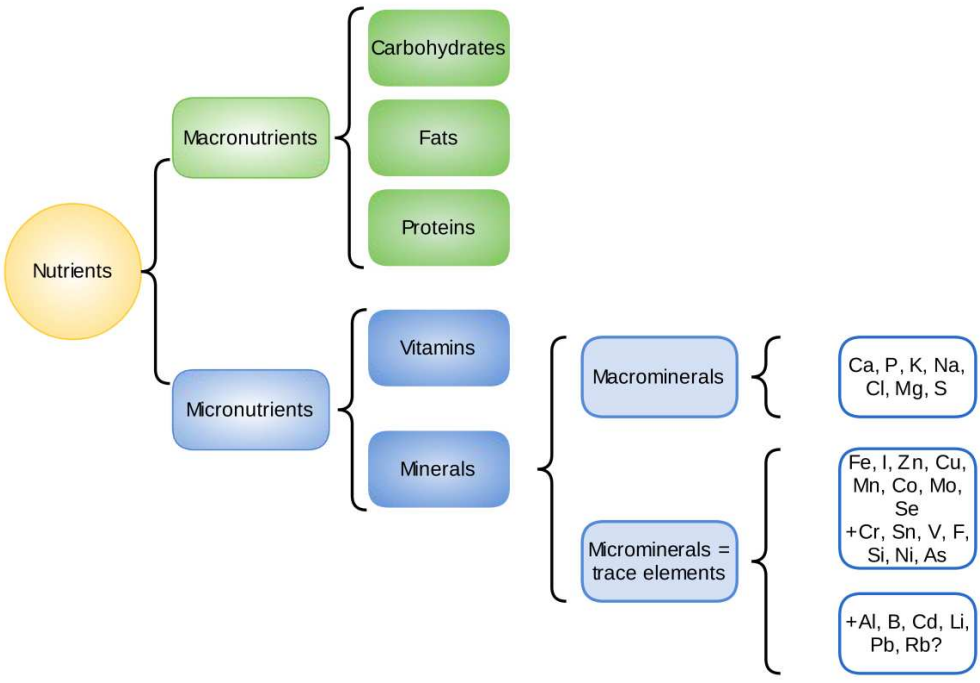

Figure 1. Essential trace elements in nutrition.

With respect to requirements, the trace elements or microminerals, form the tiniest within the smallest group, as they constitute less than 1% of the total body ash and are required in the diet at less than 1 g/kg dietary dry matter (DM), at levels far below those usually required for macrominerals (1-10 g/kg DM) (Smart & Cymbaluk, 1991).

1.2. Back to the early days of mineral and trace element research

The earliest clue for the nutritional relevance of minerals was reported by Fordyce in 1791 (Figure 2). Despite his early account on the necessity of Ca for optimal bird health, it took some additional time to fully unravel the essentiality of the trace elements. Aside from the importance of Fe and I in human nutrition, little was known about the other trace elements. It was not until the late 1920s, that another trace element was discovered to be required for optimal health (Hart *et al.*, 1928) (Figure 3).

After the discovery of Cu being an essential compound for haemoglobin synthesis, the trace element era could commence. In these early days, research mainly focussed on symptoms in farm animals. At the time of Hart's publication, in 1928, the young Eric Underwood developed a special interest in trace element research (Underwood, 1970) (Figure 4). He would eventually become one of the founding fathers of trace element research.

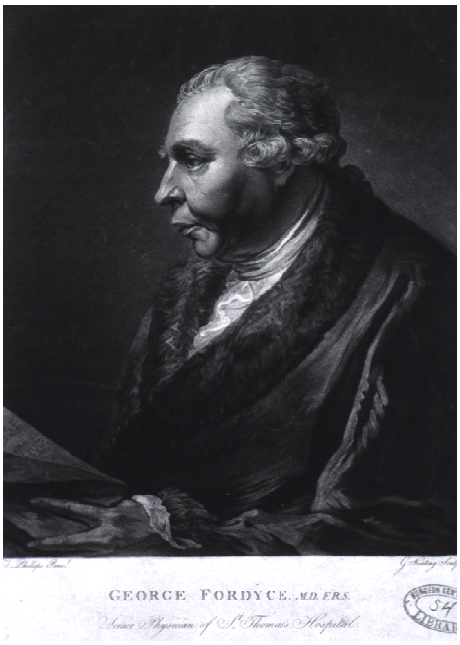

Figure 2. Portrait of George Fordyce (Courtesy of the National Library of Medicine).

"... Birds have also an evident instinct even to distinguish one kind of earth from another, as may easily be seen in Canary birds; the hen, at the time of her laying eggs, requires a quantity of calcareous earth, otherwise she is frequently killed by the eggs not passing forward properly, as I have in many instances observed, to one set of hens a piece of old mortar was given, which they broke down and swallowed, certainly not mistaking it for Canary seed, or any kind of food, but distinguishing it from a piece of brick which they did not either break down or swallow, another set at the same time were kept without any calcareous earth; many of these died, while the others, although otherwise exactly in the same circumstances, were none of them lost. It appears therefore that birds have a necessity for stones being swallowed for digestion, and earths for other purposes, and that they have an instinct which disposes them to choose the proper quantity and quality required. ..." (Fordyce, 1791)

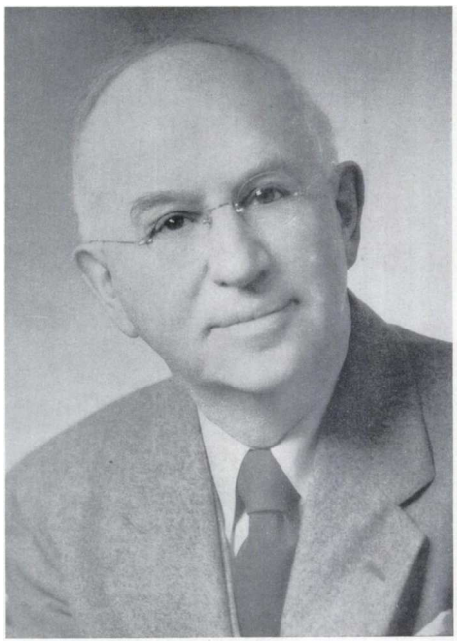

Figure 3. Portrait of Edwin Bret Hart (reproduced with permission from Elvehjem (1953)).

Shortly before (...), a trial was made of the effect of the addition of 0.25 mg. daily of copper as copper sulfate plus 0.5 mg. of Fe as ferric chloride added to our whole milk diet. This was done with an individual rat, No. 621, which had been made anemic and had a hemoglobin content of only 2.68 gm. per 100 cc. of blood (...). We made this trial of a copper salt because it fitted into our scheme of testing all inorganic elements as supplements to iron which have been recognized as present in biological materials. Further, its immediate use was suggested by the fact that we had often noticed a pale blue color in the ash of some of the materials studies, particularly the ashes of lettuce (...). Copper was also suggested by the knowledge that in some of the molluscs and crustacea it is known to exist as an integral part of the compound hemocyanin, which functions as a respiratory pigment similar to hemoglobin in the higher animals. The response in this preliminary experiment of copper sulfate feeding was indeed surprising. This preliminary experiment was with but a single animal but the effect was so convincing and helpful that we want to record (...) the weight record and hemoglobin curve of this single animal if for no other reason than its historical interest. We think that this is the first experiment in the literature giving to copper in association with iron the specific function of hemoglobin building in a mammal on a otherwise satisfactory diet. ..." (Hart *et al.*, 1928)

Decades later, a short brainstorming session during a coffee break with C. F. Mills resulted in the organisation of the first Trace Element Metabolism in Animals symposium, forming a much needed discussion forum on "the recent progress in studies on the molecular biology, metabolism and functional roles of trace elements" (Beattie & McArdle, *unpublished work*). In his introductory lecture at the Symposium, Underwood, great-heartedly admitted that his earlier statement on human nutrition, "for the overwhelming bulk of mankind a diet well-balanced and adequate in other respects is likely, on present evidence, to provide the normal individual with an abundance of all the trace elements with little chance of deleterious excess", was a massive blunder, put as if humans were immune for trace element related disorders (Underwood, 1970).

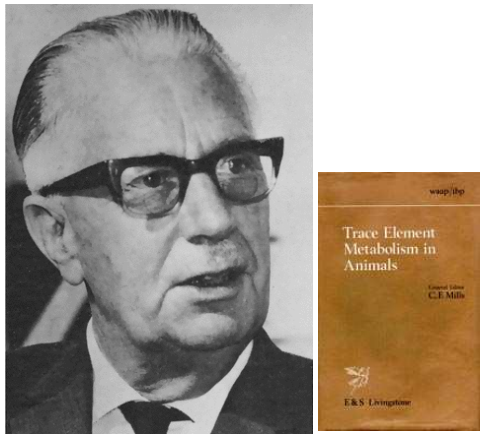

Figure 4. Portrait of Eric Johan Underwood and cover of the first TEMA symposium (reproduced with permission from Schrauzer (1980)).

Indeed, trace element research in animals, including cattle, would prove to be the herald of human nutrition research. Later on, the symposium would be renamed to Trace Elements in Man and Animals and more recently, the pendulum hit the other side, with the human research outnumbering animal research studies " (Beattie & McArdle, *unpublished work*). Nowadays, researchers call for the preservation of the crosscutting position of trace element research, in which the comparative approach remains crucial (Suttle, 2010).

1.3. What makes a trace element essential?

Until now, 15 trace elements have been reported as essential for animal and/or human health: Fe, I, Zn, Cu, Mn, Se, Co, Mo, Si, Ni, V, F, Sn, Cr and As (Table 1), although the essentiality of the last seven remains under discussion and actual requirements for these elements were not really established (NRC, 2001; McDonald *et al.*, 2002; Bender *et al.*, 2007; Suttle, 2010; Van Paemel *et al.*, 2010).

Beneficial effects of others elements, namely Al, B, Cd, Rb, Pb, Li, on animal production are known, but researchers are questioning whether or not these are truly essential (Suttle, 2010). Perhaps their benefits can be attributed merely to the concept of hormesis, in which a substance is adhering to a low-dose stimulation but high-dose inhibition pattern (Calabrese & Baldwin, 1998). Yet, their absence may not have a negative effect on normal bodily processes (Suttle, 2010).

Consequently, only some of all trace elements found in the body are actually essential. Over time, this essentiality was proven by means of controlled animal experiments with purified diets lacking the investigated element (McDonald *et al.*, 2002). The absence of a truely essential trace element affects animal health and production. Such an element exerts well-defined nutritional and biochemical roles in the body (NRC, 2005) (Table 1.).

Generally, minerals‘ functions are *catalytic* (i.e. necessary for enzyme function, e.g., Se for glutathione peroxidase), *physiological* (e.g., Na for acid-base balance), *structural* (e.g., P for bone rigidity) or *regulatory* (e.g., Ca in signal transduction) (McDonald *et al.*, 2002). For trace elements, catalytic functions are most pronounced.

Table 1. Non-exhaustive list of trace element functions and deficiency symptoms, summarizing McDonald *et al.* (2002).

Trace element	Role	Deficiency symptoms
Main		
Iron (Fe)	Haemoglobin, enzymes in electron transport chain (e.g., succinate dehyrogenase)	Anaemia
Iodine (I)	Triiodothryonine (T3) and tetraiodothyronine (T4)	Goitre, reproductive abnormalities
Zinc (Zn)	Cell replication, several enzyme systems (e.g., alcohol dehydrogenase, lactate dehydrogenase)	Loss of appetite, skin parakeratosis, low reproduction
Copper (Cu)	Ceruloplasmine, and enzymes in oxidative phosphorylation (cytochrome oxidase) and antioxidant system (superoxide dismutase)	Myelopathy ("swayback"), anaemia, diarrhoea, depigmentation of hair
Manganese (Mn)	Pyruvate carboxylase, superoxide dismutase, enzyme activator	Poor growth, skeletal abnormalities
Cobalt (Co)	Ruminally synthezised vitamin B12	Loss of appetite, pica, vague untriftiness
Selenium (Se)	A wide variety of enzyme systems, related with antioxidant system (e.g., glutathione peroxidase), hormone synthesis (type I iodothyronine deiodinase)	Myopathy ("white muscle disease"), ill thrift
Molybdenum (Mo)	Xanthine oxidase, aldehyde oxidase, sulphite oxidase	Low growth, purine metabolism disorders
Additional		
Chromium (Cr)	Glucose metabolism, possibly lipid synthesis and protein metabolism	Low growth and reproduction
Tin (Sn)	?	Poor growth
Vanadium (V)	Possibly cofactor enzymes, regulation enzyme activity (e.g., Na-K ATPase)	Low growth and reproduction
Fluor (F)	?	Poor growth, dental caries in humans
Silicon (Si)	Cross-linking agent with structural role	Bone abnormalities
Nickel (Ni)	Possibly cofactor/structural component of metalloenzymes, nucleic acid metabolism	Dermatitis
Arsenic (As)	Formation metabolites of methionine	Poor growth, rough coat

1.4. Ruminant metabolism of trace elements

Unlike other nutrients, minerals are not broken down intensively and rebuilt to metabolizable forms. The absorption of many trace elements, is carefully regulated by homeostatic control mechanisms, in an attempt to ascertain a balance between the amount of elements retained from the diet and the amount unevitably lost from the body (McDonald *et al.*, 2002).

After absorption, the micromineral is most commonly transported via the portal blood stream to the liver and other soft tissues (Figure 5). Afterwards, trace elements can be released for distribution through the systemic bloodstream to other parts of the body (Bender, 2007).

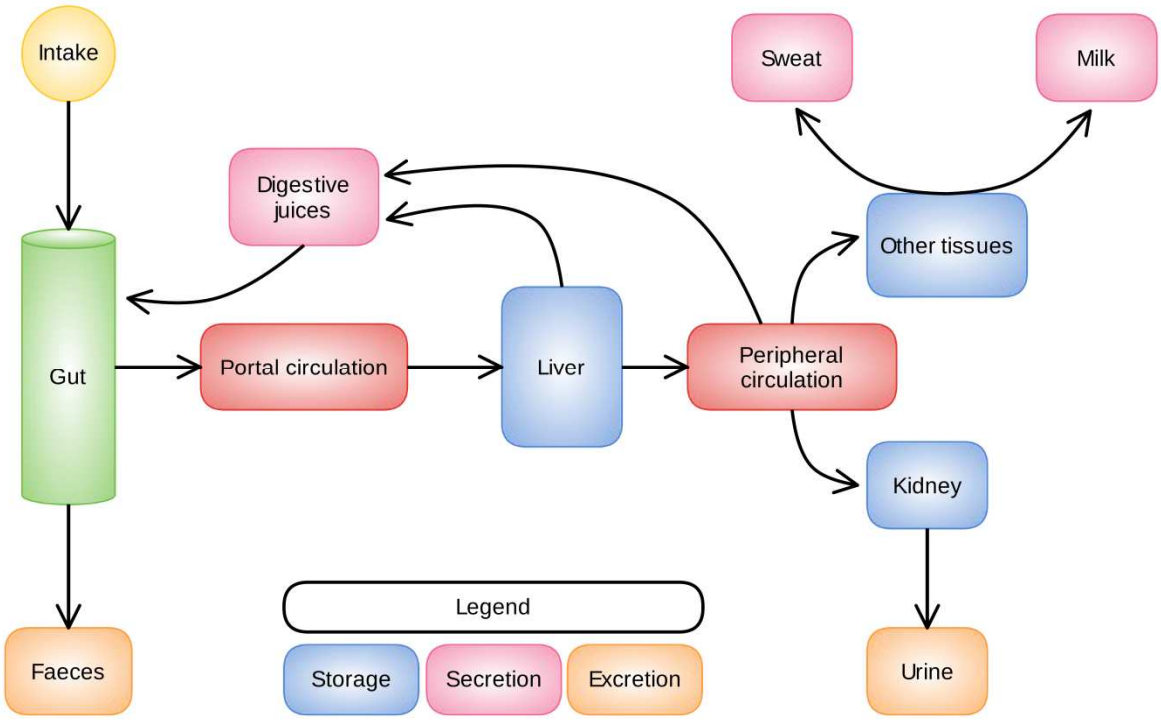

Figure 5. Mineral metabolism pathways.

Trace elements can be secreted through sweat and milk and digestive juices (e.g., saliva, bile). The latter can, if secreted at pre-absorptional locations, be recycled within in the gastrointestinal system. Excretion pathways of trace elements are faeces and urine production (Suttle, 2010).

Apart from homeostatic control mechanisms, the trace element absorption in the ruminant gut is affected mainly by other compounds present in the intestinal lumen and at the absorptive site (Smart & Cymbaluk, 1991). Even after absorption, the function of trace elements can be influenced by such compounds (Smart & Cymbaluk, 1991).

These compounds can be divided in two classes: *synergistic* (positively affecting absorption) and *antagonistic* (negatively affecting absorption) (Bender, 2007). In the context of this Introduction, we are mainly interested in the antagonists, as they can raise trace element requirements noticeably. These antagonists can be compounds of mineral nature or other dietary factors, summarized in Table 2.

Table 2. Factors negatively affecting trace element requirements for ruminants.

Trace element	Mineral antagonists[1]	Other factors[1]
Fe	-	Oxalates, phosphates, tannins[2]
I	-	Goitrogens, progoitrins and goitrins
Zn	Cu, Fe, Cd, Pb, Sn, Ca?	Tannins[2]
Cu	S, Mo, Fe, Zn, Ca, Cd	Tannins[2]
Mn	Ca, K, P, Fe	-
Co	-	-
Mo	-	-
Se	Ca, S, Zn?	-

[1]NRC (2001),
[2]Possibly affecting bio-availability of trace elements, based on data from Gillooly *et al.* (1983) and Karamać (2009)

1.5. On the search for bovine trace element requirements

The most commonly used methods to establish bovine trace element requirements are the factorial method and the dose-response method (Bender, 2007).

The *factorial method* calculates the net and gross requirements based on data typical for a certain physiological stage. The net requirements are defined as the levels necessary for the maintenance of normal physiological functions, increased by those additionally required for optimal growth, production (e.g., muscle growth, milk production),

reproduction (e.g., foetus development) and extensive work (e.g., ploughing labour). These levels are calculated by adding the amount of the elements retained and the amount lost endogenously from the animal body (NRC, 2001).

Furthermore, in the factorial method, the gross requirements can be estimated from the net requirements by dividing these by the true absorption coefficient. The latter expresses the percentage of the consumed diet truly retained from digestion, taking into account the intestinal absorptive capacity and potential absorbability of the mineral (Bender, 2007). Obviously, those elements with a lower absorbability will require higher dietary requirements and will have a higher safe allowance than those with a high absorbability (Suttle, 2010).

The *dose-response method* works differently, and is sometimes used in case the true absorption coefficient is difficult to establish, the bodily disposition of the element is closely related to the intake, even when requirements are met, or other technical problems arise (NRC, 2001; Suttle, 2010). This method employs dietary trials with different groups of animals fed with a range of levels of the particular element and evaluates the general performance, or differences in specified response variables (e.g., ceruloplasmine for Cu) (Meschy, 2000).

The currently established trace element requirements and maximum tolerable concentrations, established for *Bos taurus* cattle are presented in Table 3. As mentioned above, no requirements for the other essential or possibly essential trace elements are presently available (Van Paemel *et al.*, 2010).

Table 3. Guidelines for *Bos taurus* trace element supply (mg/kg DM)

	Requirements		
	Beef cattle[1]	Dairy cattle[2]	MTL[3]
Cu	10.0	11.0	40.0
Fe	50.0	12.3	500
Zn	30.0	43.0	500
Mn	20.0	14.0	2000
Se	0.100	0.300	5.00
Co	0.100	0.110	25.0
I	0.500	0.600	50.0
Cu:Mo[4,5]	>1-3	>1-3	
Fe:Cu[4]	<50-100	<50-100	

[1]NRC, 2000
[2]Adult Holstein cow with production of 25 kg milk (NRC, 2001)
[3]MTL= maximum tolerable levels (NRC, 2005)
[4]Advisory levels for Cu antagonists (Suttle, 2010)
[5]Provided that dietary S > 2 g/kg DM and dietary Mo < 15 mg/kg DM (Suttle, 2010)

1.6. Meeting cattle requirements and establishing an adequate trace element status: the soil-plant-animal flow

What Underwood only realised at a later stage on trace elements in human nutrition, animal nutritionists knew long before: the natural cattle diet is far from flawless in establishing an adequate trace element status. Many factors affect the flow of trace elements from soil through plant to the animal, and even in case of dietary trace element concentrations in line with established requirements, other influences can cause variation in trace element status response (Figure 6).

The amount of trace elements present in the soil is predominantly influenced by the parent material and its typical mineral character (Jumba *et al.*, 1995; Thornton, 2002). The "availability" of these soil minerals and trace elements for plant uptake is influenced by the geochemical character, pH and drainage of the soil. As the soil pH lowers, plant Mo uptake lowers whereas Co, Mn and Zn uptake increases (Suttle, 2010),

whereas waterlogging induces a change in trace element mobilisation in the flooded soils, causing an increased plant uptake of, e.g., Mo (Du Laing *et al.*, 2009).

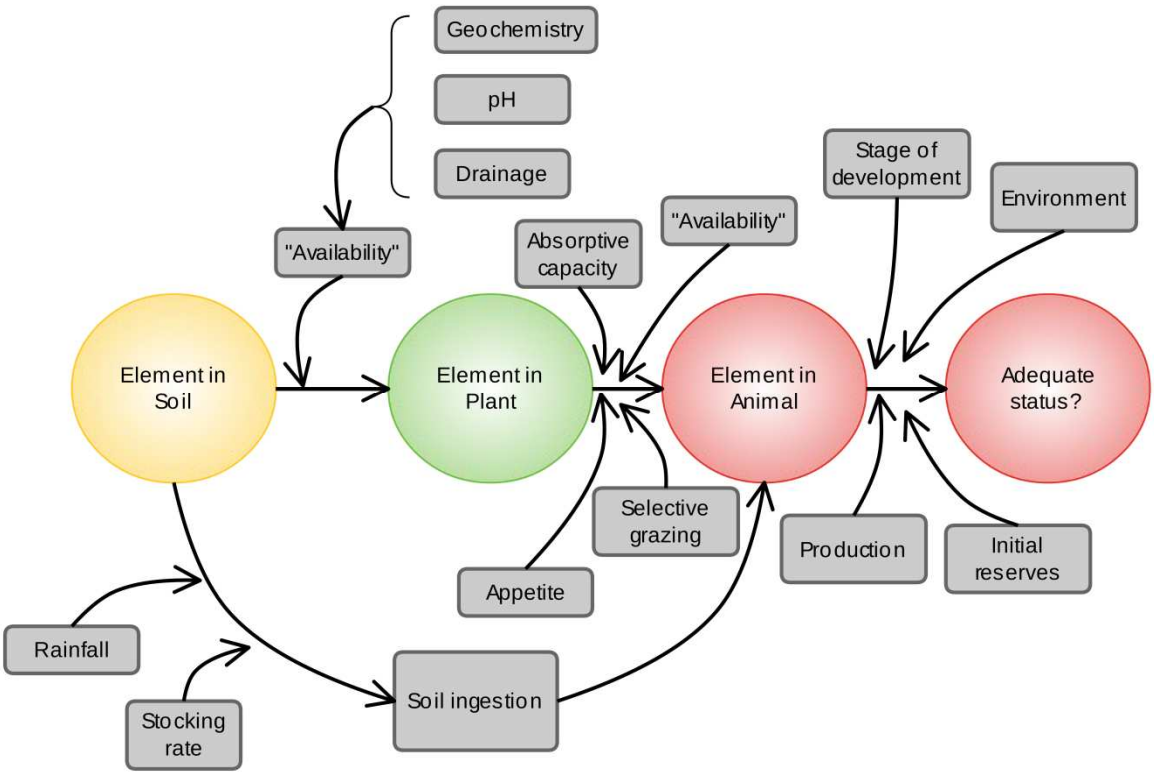

Figure 6. The soil-plant-animal flow of trace elements and established factors affecting this flow (redrawn with permission from Suttle (2010)).

Worldwide, forage trace elements most commonly present in concentrations inadequate for cattle nutrition, are Co, Cu, I, Se and Zn (McDowell, 1996). However, forages are also known to contain very variable amounts of trace elements (Abdelrahman *et al.*, 1998; Faye *et al.*, 1983; Gizachew *et al.*, 2002). Obviously, plant genus and type (herbaceous vs. grasses) will explain some of the variation in trace element content (Jumba *et al.*, 1995). Other factors playing a role are the stage of maturity of the plant, with reduced trace element content as the plant matures (McDowell, 1996) and season, with generally lower trace element concentrations in the dry season (Pastrana *et al.*, 1991).

Next to direct dietary supply, soil ingestion can constitute 2 to 20% of dry matter intake in grazing cattle (Thornton, 2002). Due to the higher amounts of trace elements in the soil in comparison with the plants growing on it, this soil uptake can be both beneficial

as disadvantageous for the grazing animal (McDowell, 1996), a welcome supplement or a poisoned gift. For instance, soil ingestion is known to exacerbate the Cu status because of the large amount of Fe present in these particles (Suttle et al, 1984).

Apart from initial storage, dietary intake, dietary antagonists and physiological stage affecting requirements, other factors influence the eventual trace element status and response to trace element supply. Generally, with increasing age, trace element concentrations in transport pools are also increasing, with the exception of Co, higher in neonates. On the other hand, the liver storage only slightly increases with increasing age, but not for Se and Cu, of which normal liver concentrations are much higher in neonates than in adults (Herdt & Hoff, 2011). Furthermore, male animals could have a higher trace element status than female animals (Miranda *et al.*, 2007), indicating a certain sex specific status, as seen for Cu in humans (Milne, 1998). Also, increasingly, studies are reporting on the possible presence of a breed specific sensitivity towards trace element imbalance. More specifically, evidence is piling up on a disparate proneness to Cu deficiency in the Simmental and Angus cattle breeds (Ward *et al.*, 1995; Mullis *et al.*, 2003; Fry *et al.*, 2013). Consequently, trace element requirements of such breeds might differ, but this was not formally established yet.

1.7. How to evaluate the trace element status in cattle

1.7.1. General considerations on trace element diagnostics

The best diagnosis for inadequate trace element supply is a good response to supplementation (Clark *et al.*, 1985). However, trial supplementing of an entire herd is often not feasible, very costly or not efficient (Olson, 2007). Therefore, it is equally important to design thresholds values or ranges for deficiency based upon responsive tissues, for decision making. The evaluation of bovine trace element status is most commonly performed by means of the determination of concentrations in either liver and/or plasma samples (Kincaid, 2000; Puls, 1988; Suttle, 2010) (Table 4 & 5).

Table 4. Threshold values for trace element deficiency in liver and serum of *Bos taurus* cattle.

	Liver (mg/kg DW)[1]			Serum (mg/l)		
	Suttle (2010)	Kincaid (2000)	Puls (1988)	Suttle (2010)	Kincaid (2000)	Puls (1988)
Cu	19	33	1.75-35	0.57[a]	0.50[a]	0.02-1.20
Mo[2]	_	_	7.0-3500	_	_	0.10-10.0
Fe[2]	1000	159-2100	186-2450	0.6-1.8	4.0-6.0	4.0-6.0
Zn	_	<20-40	70-140	0.40-0.60	0.20-0.40[a]	0.20-0.60
Mn	9.0	7.0	3.5	0.020	0.005	0.005
Se	0.05-0.07	0.10-0.50	0.07-0.60	0.008-0.009	0.060[b]	0.002-0.060
Co	-	_	0.175	_	_	0.090[b]
I	_	_	_	0.030-0.040	0.010-0.050	0.010-0.050

[1]Conversion factor FW to DW: x 3.0 (Suttle, 2010), and x 3.5 (Puls, 1988), as advised by these authors
[2]Mo and Fe: concentrations indicating excess of these Cu antagonists
[a]Plasma,
[b]Whole blood

Diagnostic thresholds and reported normal concentrations range widely between authors, rendering decision-making often difficult (Table 4 & 5). Further, often grey zones of sub and supraoptimal concentrations exist within reported values of an author, of which it is not known whether or not these are harmful for cattle on the long term and hence, whether or not intervention is needed (Puls, 1988). This is particularly true for the evaluation of Se status, where further research on the topic is needed. Furthermore, little is known about the wide applicability of these thresholds, as they might not take normal variations among and between breeds into account.

Table 5. Reference ranges for trace elements in liver and serum in *Bos taurus* cattle.

	Liver (mg/kg)DW)[1]	Serum (mg/l)[1]
Cu	50-600	0.6-1.1
Mo	1-4	0.002-0.035
Fe	140-1000	1.1-2.5
Zn	90-400	0.6-1.9
Mn	5-15	0.0009-0.0060
Se	0.7-2.5	0.065-0.140
Co	0.10-0.40	0.00017-0.002
I	-	-

[1]Herdt & Hoff, 2011

1.7.2. Liver samples

For most elements, the liver is considered the main storage pool and responsive to dietary supply (Herdt & Hoff, 2011; Ouweltjes, 2007; Suttle, 2010). The reaction of this main storage pool during different phases occurring in case of deficiency and overload is visualized in Figure 7. Due to the above mentioned characteristics, liver sampling is regarded as the most precise method to evaluate trace element status in the bovine body. For Se, kidney concentrations are higher than liver concentrations (Puls, 1988) and the storage of the element Zn seems to be rather evenly distributed over different tissues, yet, for both elements, liver is still the best choice sample because of its higher responsiveness to dietary intake (Suttle, 2010).

Nevertheless, liver biopsy is still not commonly performed, due to, although rare, some health risks involved with this sampling method, such as clostridium infections, and pneumothorax (Vermunt, 2011). Therefore, the search for more easy-to-sample indicators is open.

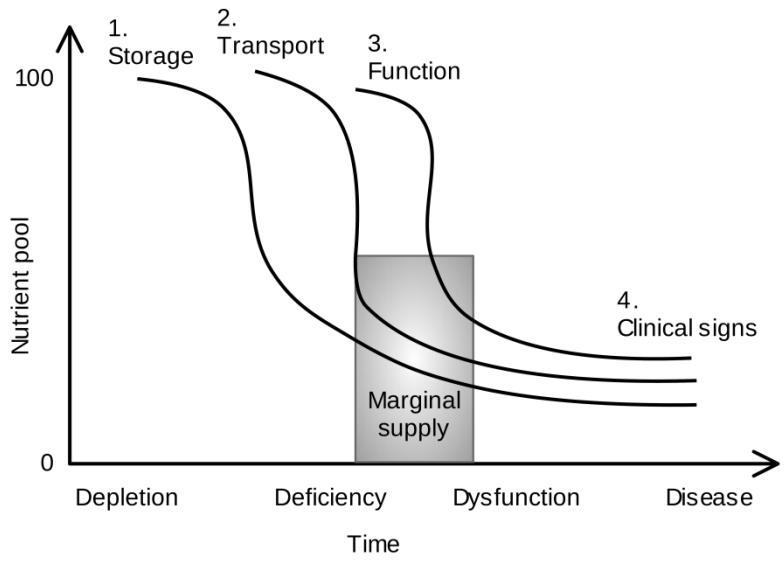

Figure 7. The sequence of phases occurring in mineral deprivation. These phases are related to curvilinear changes in trace elements pools responsible for storage (e.g., liver), transport (e.g., plasma) or functional (e.g., muscle enzyme) purposes. For all elements, there is a marginal area, with nearly exhausted stores and the onset of failure of mineral-dependent functions, but where animals seem healthy from outwards (redrawn with permission from Suttle (2010)).

1.7.3. Plasma samples

In contrast to liver sampling, plasma sampling is useful as a more practical way to gain insight in the trace element status of cattle and to quickly screen large numbers of animals and herds (Herdt & Hoff, 2011). However, some factors falsely reduce or elevate plasma concentrations, such as inflammation (↑ Cu: Laven *et al.*, 2007; ↓ Zn: Orr *et al.*, 1990), gastro-intestinal parasites (↓ Cu: Adogwa *et al.*, 2005), heat stress (↓ Zn: Wegner *et al.*, 1973) and partus (↓ Zn: Goff & Stabel, 1990). Therefore, in case of aberrant plasma trace element concentrations, the presence of such factors should be investigated.

Also, depending on the specific metabolism of the trace element, plasma concentrations might have a different meaning. For most elements, plasma concentrations, as part of the transport pool, will react slower to deficiency than storage values (e.g., Cu) (Figure 7). For elements with small or slowly mobilized stores, transport and storage pools (e.g., Zn) will react simultaneously. For these elements, strong homeostasis mechanisms might be present in an attempt to tightly control plasma concentrations of trace elements. For instance, in the case of Zn, intestinal absorption is carefully regulated by Zn intake (Hiers *et al.*, 1968) and plasma concentrations therefore, remain fairly constant, until the border of these homeostasis mechanisms is reached, in case of extremely high or low intake. For others, plasma might even serve as the storage pool (e.g., Co) (Suttle, 2010). Such patterns of homeostasis, storage and transport might cause a misinterpretation of the animal status based on plasma concentrations (Herdt & Hoff, 2011). Still, when including some caution in evaluating trace element concentrations and applying them merely for screening methods, blood continues to have its role in trace element diagnostics.

In order to evaluate the value of blood sampling or any alternative method to investigate trace element status of cattle, supplementation trials with rising amounts of dietary supply could be utilized, or one could analyse whether or not changes in blood concentrations relate well to main storage tissue status changes, either using correlation/regression or dichotomized approaches (Claypool *et al.*, 1975; Minatel & Carfagnini, 2002).

1.8. Investigating the effects of trace element status

1.8.1. Introduction

Trace element research initiated from case reports on severe symptoms related with deficiency or direct toxicity. Later on, studies elucidated the role of trace elements as cofactors in many important enzymes (Suttle, 2010). However, the background of some symptoms, such as severe diarrhoea in Cu deficiency, as well as the effect of deficiency on other bodily functions, such as reproduction, is not fully unravelled yet (Wichtel, 2003). Furthermore, the effects of prolonged marginal deficiency, as a less extreme point on the mineral range from severe deficiency to severe toxicity, are not well known. Therefore, research is still needed on the effects of trace element status (deficiency or excess) on the animal metabolism, in both controlled experiments and on-farm trials.

Within the context of this work, we will focus on the effects on animal health, as expressed by optimal parameters of immunity, anti-oxidant status and disease resistance, and production, i.e. weight gain and carcass appraisal in beef cattle and milk and component yields in dairy cattle, both more generally defined as *performance*, in addition to those influencing human nutrition, i.e. the impact of bovine trace elements status on trace element supply through *animal products*. Reproduction was not included, as we defined it out of the scope of this work.

1.8.2. Considerations on trials investigating impact of trace elements

Supplementation proves to be an effective way to raise trace element status when the supply is inadequate (McDowell, 1996) and is, consequently, an appropriate research tool to investigate effects of trace element status. However, literature data reveal a very heterogeneous group of supplementation trials: organic (Givens *et al.*, 2004; Rabiee *et al.*, 2010) versus inorganic (Sharma & Joshi, 2005; Sharma *et al.*, 2005) trace element

supplementation, with (Bailey *et al.*, 2001; Sprinkle *et al.*, 2006) or without (Uchida *et al.*, 2001; Cortinhas *et al.*, 2010; Cortinhas *et al.*, 2012) control groups, single (Kumar *et al.*, 2006; Mandal *et al.*, 2007) versus multiple (Ahola *et al.*, 2004; Hackbart *et al.*, 2010; Dang *et al.*, 2013) trace element supplementation, and a wide definition of the concept "control", ranging from below requirements (Engle *et al.*, 2001; Scaletti *et al.*, 2003) to concentrations doubling these requirements (Campbell *et al.*, 1999; Lamb *et al.*, 2008; Sobhanirad *et al.*, 2010). While all these studies have merit, conclusions should be stated with care. Overall, this wild panache of studies may seem to produce conflicting results and may confuse future scientists as well as practitioners. The critique is rising on such research practices as some authors call out for a nuanced assessment of potentially commercially biased experiments (Suttle, 2010), and others advocate a more standardized (Weiss & Spears, 2006) and combined research approach, both mechanistic and applied (Wichtel, 2003).

As an alternative for the supplementation trials, new studies are coming up investigating the link between the presence of disease on farm level and trace element deficiencies, e.g., through odds ratios (Guyot *et al.*, 2009; Machado *et al.*, 2013). Furthermore, the association between a certain status and a continuous outcome (using regression and/or correlation coefficients) can also be investigated. Finally, the unbeaten track of systematically reviewing the effects of trace element supplementation could be a promising option to provide more generalized views on the topic.

1.8.3. Performance effects of trace element status

1.8.3.1. Anti-oxidant status, immunity and disease resistance

In the context of this work, we consider health as optimal response towards oxidative stress and a good immunity. Trace elements are involved in all these processes, which are also interrelated.

Oxidative stress is defined as an imbalance between pro-oxidants and anti-oxidant systems in the body, with a final overload of reactive oxygen species (ROS) capable to

initiate lipid peroxidation and disrupt cell walls with cell death as a consequence (Davies, 2000). If not restrained, the rampant production would lead to loss of vital organ functions and eventually, animal death (Davies, 2000). Luckily, the body has a broad range of tools to tackle such dangerous compounds. Trace elements perform important tasks in this *anti-oxidant system,* as cofactors of enzymes. Two types of superoxide dismutases (SOD), the cytoplasmatic or extracellular Cu,Zn dependent SOD and mitochondrial Mn related SOD, as well as the Se dependent glutathione peroxidase (GSH-Px), help fight the oxidative burden in ROS reduction reactions (Spears & Weiss, 2008). Other selenoproteins such as selenoprotein P and five different isoforms of GSH-Px, are also thought to perform such tasks (Sordillo & Aitken, 2009). Furthermore, the molecules ferritin and ceruloplasmin (Cp), Fe and Cu dependent respectively, protect the body against metal-mediated oxidation reactions (Davies, 2000).

Immune cells are very susceptible to oxidative stress since their membranes contain high levels of polyunsaturated fatty acids (PUFA). The ROS, however, are also formed as a part of the *immune response*, in order to effectively kill host intruders (Spears & Weiss, 2008). Within the immune system, trace elements seem to perform important tasks based on deficiency symptoms, yet, their true function is ill-defined. For a extended overview of current knowledge on these functions in humans, readers are encouraged to read the review of Wintergerst *et al.* (2007).

Less research on the subject has been performed in ruminants, although some data suggest marked differences with human subjects. The combined work of Jerry Spears and William Weiss (Spears, 2000; Weiss & Spears, 2006; Spears & Weiss, 2008) nicely compiled current knowledge on immune status effects of trace element supplementation in ruminants. Briefly, they stated that Cu supplementation seems to have a positive effect on neutrophil killing function, but studies investigating effects on cellular and humoral immunity are less consistent (Spears, 2000). For Se, on the other hand, it is very clear that supplementation, alone or in synergistic combination with vitamin E, has a positive influence on immune function, both on phagocytic and specific immunity, and hence, resistance towards mastitis (Weiss & Spears, 2006). The authors also suggest that the impact of Zn supplementation on immune functions is usually minor in ruminants, at least based on study results at the moment. Zn, however, does

have an important role in replication of immune cells as well as for integrity of the intestinal barrier (Spears, 2000; Weiss & Spears, 2006). It seems that much more is to be unravelled in this field of research.

Theoretically, we expect a strengthened *disease resistance* in cattle with an improved trace element status as a result of improved immunity and anti-oxidant status. Selenium is renowned for its positive influence on susceptibility to intramammary infections with reduced California Mastitis Test (CMT) scores, expressing the presence and degree of subclinical mastitis, whereas reported effects of Cu are inconsistent (Spears, 2000). Despite the ill-defined role of Zn in ruminant immunity, the element is able to positively affect disease resistance (Spears, 2000). Field trials on beef and dairy farms also linked the deficient status of these elements with increased odds ratios for disease (Zn, Cu and Se: Enjalbert *et al.*, 2006; Zn, Cu, Se and I: Guyot *et al.*, 2009).

1.8.3.2. Production

Production is the main objective of animal husbandry and it is therefore important to measure the impact of trace elements on production parameters. Optimal production is the result of many factors, including genetic potential, disease burden, nutrition and management. The adequacy of the trace element supply only is a small facet in a broader picture. However, whether trace element deficiency can have noticeable impact on animal production is not fully unravelled.

In beef cattle, earlier research investigated the effect of trace element supplementation on live production characteristics with sometimes conflicting results. Spears & Kegley (2002) found a positive effect of Zn supplementation on average daily gain (ADG) in the growing, but not in the finishing phase of *beef cattle*. These authors also found a positive effect on quality grade and marbling grades as well as a tendency for positive effects on yield grade and back fat thickness. Manganese supplementation on the other hand, did not cause an effect on any live performance parameters or on carcass appraisal (Legleiter *et al.*, 2005). Furthermore, for Se, Droke & Loerch (1989), did not observe any influence of supplementation on production characteristics.

In the trial of Ward & Spears (1997), Cu supplementation tended to positively affect ADG, and was also associated with leaner carcasses, with a lower yield grade and hot dressing percentage and a trend for lower back fat percentage. In the presence of high levels of the Cu antagonists Mo and Hansen *et al.* (2008) also found a higher ADG, dry matter intake (DMI) and Gain:Feed ratio in Cu supplemented cattle versus control animals. Bailey *et al.* (2001), García-Vaquero *et al.* (2011) and Ward *et al.* (1993), on the contrary, did not find any effect of Cu and Engle & Spears (2000) even observed a decreased performance in Cu supplemented steers during the finishing phase. The effects of other important trace elements as well as the effect of a complete trace element mix on beef cattle performance are not investigated well.

Further, milk yield and milk component (fat, protein, lactose) yield in *dairy cattle* are generally not reported to be affected by the trace element status of the animal. Indeed, Engle *et al.* (2001) did not observe differences in milk production with or without Cu supplementation whereas Engel *et al.* (1964) did not observe an effect on component yields. Also, no effects of Se and Zn were found by Juniper et al (2006) and Sobhanirad *et al.* (2010), respectively. The effects on milk and milk component yield were not examined for other trace elements or for a complete trace element mix. Enjalbert *et al.* (2006), however, reported an increased odds ratio for low milk production (as reported by the dairy farmer) in Zn deficient cattle.

1.8.4. Trace elements in animal products

1.8.4.1. Introduction

Next to their role for animal performance and health, trace elements in animals can also fulfil a role in human nutrition, through their storage and secretion in animal products, such as milk, meat and organs. The most complete diagnostic data on mineral levels in these products, for animal health were provided by Puls (1988) and are presented in Table 6.

Table 6. Adequate range for trace elements concentrations in bovine tissue (mg/kg WW) and milk (mg/l) according to Puls (1988)

Organ	Cu	Mo	Fe	Zn	Mn	Se	Co
Liver	25-100	0.14-1.40	45-300	25-100	2.5-6.0	0.25-0.50	0.02-0.09
Kidney	4.0-6.0	0.22-0.57	30-150	18-25	1.2-2.0	1.0-1.5	0.071
Muscle	1.2-1.5	-	10-12	30-70	0.44-0.84	0.07-0.15	-
Heart	-	-	-	-	-	-	-
Milk	0.05-0.60	0.028-0.12	0.2-6.3	2.3-7.5	0.02-0.07	0.03-0.05	0.04-1.10

Guidelines for required human trace element supply are summed in Table 7. Within this context, the word "adequacy" has a twofold meaning, adequate as in supplying enough for optimal animal health and performance, and adequate as in providing a substantial amount of trace elements for human nutrition.

Table 7. Recommendations for human trace element intake

	FNB, IM (2000), RDA[1,2]		WHO/FAO (2002), RNI[3]	
	Woman	Man	Woman	Man
Se, µg/d	55	55	26	34
Cu, µg/d	900	900	-	-
Mn, mg/d[4]	1.8	2.3	-	-
Zn, mg/d	8	11	3-10[a]	3-10[a]
Fe, mg/d	18	8	20-59[a]	9-27[a]
Mo, µg/d	45	45	-	-
I, µg/d	150	150	150	150

[1,2]Food and Nutrition Board, Institute of Medicine (2000a,b): RDA = recommended daily allowance,
[3]World Health Organization/Food and Agriculture Organization of the United Nations (2002): RNI = recommended nutrient intake,
[4]AI= adequate intake,
[a]Range depending on bio-availability of micronutrient

1.8.4.2. Organs and meat

Overall, few data are present on *trace element concentrations* in bovine organs and meat. Most research was performed in polluted areas with a focus on contamination of soils with heavy metals (Sedki *et al.*, 2003; Oyaro *et al.*, 2007; Miranda *et al.*, 2009; Waegeneers *et al.*, 2009). Also, the true value of the reported ranges of adequacy of Puls (1988) for assessing trace element status of these tissues is not well investigated. Doyle and Spaulding (1978) reviewed data on trace elements in cattle tissue and they found trace element concentrations widely inconsistent with adequate ranges of Puls (1988),

especially for Fe, Zn and Mn muscle concentrations (Table 8). Indeed, especially in the case of muscle concentrations, literature comparison is difficult, as rarely the type of muscle is mentioned, and different types of muscles differ in trace element concentrations (García-Vaquero *et al.*, 2011).

As mentioned above, liver is the main storage organ for many trace elements, whereas the kidney often forms the second largest storage pool in the body (Suttle, 2010; Herdt & Hoff, 2011). Based on the normal tissue ranges stated in Table 5, this is particularly true for Cu, Mo, Fe and Mn. For Zn and Co, normal liver concentrations are high, but do not differ much from muscle and kidney levels, respectively (Puls, 1988). The storage of Co is still poorly understood, due to its role as integrated part of the cobalamin metabolism (Stangl *et al.*, 1999), whereas the more diffuse storage of Zn is rather an indication of a poorly developed storage system, with low bodily reserves (Zn) (Miller, 1969). Unlike for the other elements, the kidney contains the highest Se concentrations instead of liver (Puls, 1988). Adequate liver concentrations of Cu, Mn, Fe, Zn are much more variable than those of Se and Co (Puls, 1988). For most elements, kidney concentrations do not seem very variable, with the exception of Fe, and trace element muscle concentrations reported by the same author, also seem fairly constant (Puls, 1988). Recently, authors reported that trace element concentrations in more active muscle types (e.g., diaphragm and cardiac muscle) with less fat seem to be higher than in other muscle types (e.g., pectoral and semitendinosus muscle) (García-Vaquero *et al.*, 2011).

Table 8. Reported means of trace elements concentrations found in bovine tissue (mg/kg WW) as reviewed by Doyle & Spaulding (1978)

Organ	Cu	Mo	Fe	Zn	Mn	Se	Co
Liver	44-100	_	51-81	26-44	2.3-3.5	0.12-1.15	_
Kidney	4.8-5.6	_	69	13-22	0.75-1.25	1.17	_
Muscle	1.2-2.7	_	20	19-31	0.21-0.23	0.05-0.09	_
Heart	3.7	_	64	14-22	0.29-0.74	-	_

There is still a dearth on information on the *impact of trace element status* on trace element concentrations in organ and muscle tissue as, in fact, little knowledge is present on how the trace element distribution to tissues is affected, during deficient, sufficient and toxic supply periods. The liver is known to be the most responsive organ

to an altered trace element status for most elements (Ouweltjes *et al.*, 2007). This is particularly true for Cu, Fe and Se, the latter despite having higher kidney concentrations. For Zn, Co and Mn, liver concentrations will alter in case of extreme changes in trace element status (i.e. toxicity or deficiency) but they are less responsive to smaller variations in intake and status (Suttle, 2010). Theoretically, the kidney is expected to react in line with liver, as the second most important organ of storage, and bodily detoxification. However, this is only true, and to a lesser degree than for liver concentrations, for Cu, Fe, Se and Mn (Suttle, 2010). The impact of trace element status on trace element disposition in muscle is also not fully unravelled. García-Vaquero *et al.* (2011) suggest that in the case of adequate trace element supply, internal homeostasis mechanisms are predominantly affecting trace element storage. During an initial phase of depletion, the body often exerts a higher distribution of trace elements to soft tissues with an increase or limited reduction of muscle concentrations (Zn: Miller, 1969; Fe: Suttle, 2010). After prolonged deficiency, muscle concentrations seem to deplete eventually (Cu: Mills *et al.*, 1976). Other studies with generally low trace element supply, showed that liver status and muscle storage were linearly related for at least some elements (Se: Pavlata *et al.*, 2001).

1.8.4.3. Milk

As compared with tissue concentrations, the trace element content of bovine milk in cattle with an adequate status is rather low for Cu and Mn, whereas high for Fe, Se, Zn and especially Mo (Puls, 1988). Unlike in muscle, *trace element concentrations* in milk are also quite variable (Puls, 1988).

With regard to the *effect of trace element status* on secretion of trace elements in milk, for some elements, such as Cu, it is known that deficiency will cause a drop in milk concentrations. As such, milk concentrations of Zn and Cu can be increased by supplementation to normal concentrations but only if prior dietary levels were deficient (Zn: Miller, 1970; Cu in dams: Suttle, 2010). Milk Se and Co concentrations can be largely increased, without reaching a plateau even when adequate dietary amounts are fed (Se: Knowles *et al.*, 1999; Co: Kincaid & Socha, 2007). Although other factors, such as processing can also affect trace element concentrations (Coni *et al.*, 1995) and the

association with trace element status is not well understood, dietary intake seems to affect milk concentrations, at least for some elements.

2. Trace element supply and cattle production in tropical regions

The tropics are defined as the zone which is limited by the tropics of Cancer in the North and the tropics of Capricorn in the South (El-Swaify *et al.*, 1982). Most tropical countries have an arid or tropical type of climate with high temperatures and either extremely low (e.g., Sudan, Somalia) or extremely high (e.g., Burundi, Vietnam) annual precipitation levels respectively, whereas in others, inclusions with a rather temperate climate exist (e.g., Zambia, Ethiopia) (Peel *et al.*, 2007). Many soils found in these areas are characterized by low nutrient levels (e.g., Ferralsols, Nitisols) and/or suffer from waterlogging (e.g., Vertisols) (Dudal, 1980). As explained above, intrinsically, due to certain parent materials, soils in tropical areas often contain low levels of some trace elements, combined with toxic levels of others (Dudal, 1980; Faye *et al.*, 1991). Moreover, tropical soils are prone to erosion, i.e. the weathering of the land surface through forces of nature, such as water and wind and often facilitated by inappropriate land management (Dudal, 1980). Hence, trace elements are leached away (Olife *et al.*, 2007), leaving even more impoverished grounds behind (Pimentel, 2006). Pastures growing on these soils, frequently exhibit low levels of trace elements, e.g., of Cu, Zn, Se and Co, whereas Mn and especially Fe concentrations below cattle requirements are very uncommon (McDowell & Arthington, 2005). However, the trace element content of tropical pastures is equally variable as in non-tropical areas.

Cattle in the tropics are most often extensively kept and free-ranging on communal grazing lands. The major constraints to cattle production in the tropics are the stressful climate and high disease burden but also the presence of low quality feeds with low

energy and protein levels and often imbalanced trace element supply, as mentioned earlier (Leng, 1990; McDowell & Arthington, 2005). Due to their robust nature, the most commonly used cattle in the tropics are zebu cattle (Turner, 1980) (Figure 8).

Figure 8. Zebu oxes ploughing the land at the Gilgel Gibe catchment, Ethiopia

3. Zebu Cattle

3.1. Domestication waves of cattle

Generally, two schools of thought exist on the origin of domesticated cattle, classified collectively as *Bos primigenius* (MacHugh *et al.*, 1997). One school postulates that all cattle originate from the same ancestor: the aurochs, *Bos primigenius primigenius*. According to this school, the humpless "taurine" type was domesticated first from the feral type around 8000 and 9000 years ago in the Near East. Afterwards, the humped "zebu" or "indicine" type developed through selection and breeding (Epstein, 1971). Nonetheless, another school, which is most widely supported, states that the two types descend from different ancestors, diverging at least 200 000 years ago (MacHugh *et al.*, 1997). They seemed to be domesticated at separate places, the taurine type would indeed descend

from the *Bos primigenius primigenius* around 10000 years ago at the Fertile Crescent, whereas the zebu type would descend from the *Bos primigenius namadius*, and was domesticated around the Indus valley some 2000 years later (Loftus *et al.*, 1994; Caramelli, 2006; Ajmone-Marsan *et al.*, 2010) (Figure 9).

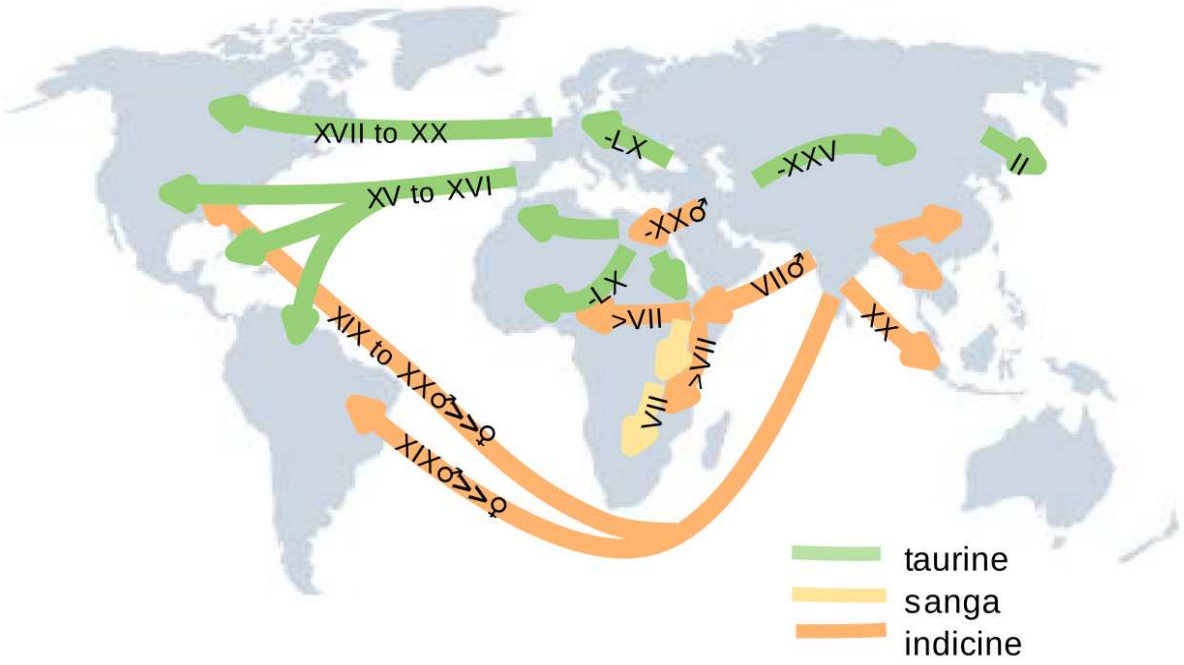

Figure 9. Cattle migrations worldwide. Roman numbers depict the century of migration. Migrations within Europe, as well as to Australia are not included. Reproduced with permission from Ajmone-Marsan *et al.* (2010).

Unmistakenly, the first cattle setting claw on African ground were taurine-typed (Bradley *et al.*, 1998; Hanotte *et al.*, 2000). They arrived there around 8000 years ago, at the same time of taurine introduction in Europe (Ajmone-Marsan *et al.*, 2010). Much later, around 2500 years ago, zebu cattle were introduced for the first time to the continent. Nevertheless, these newcomers exerted little impact on the large taurine population, although some local crossbred products did arise from their arrival, namely the sanga (a crossbred of the African zebu and indigenous taurine cattle) and zenga (crossbred of the African zebu and sanga) (Hanotte *et al.*, 2000), although others contest this (Grigson, 1991). One way or the other, the Arab invasion some 700 years before Christ would leave a much more marked influence on the African cattle population. Large groups of mainly male zebu cattle were introduced and quickly penetrated the

African population, due to their robustness and distinct tolerance towards rinderpest infestations (Ajmone-Marsan *et al.*, 2010). This would result in a remarkable sex-driven zebu introgression in the African cattle population, with a high percentage of zebu Y-chromosome alleles (originating from the bulls) and a high percentage of zebu autosomal admixture but hardly any maternally dependent zebu mitochondrial DNA (mtDNA) (Suzuki *et al.*, 1993; MacHugh *et al.*, 1997; Hanotte *et al.*, 2000) (Figure 10).

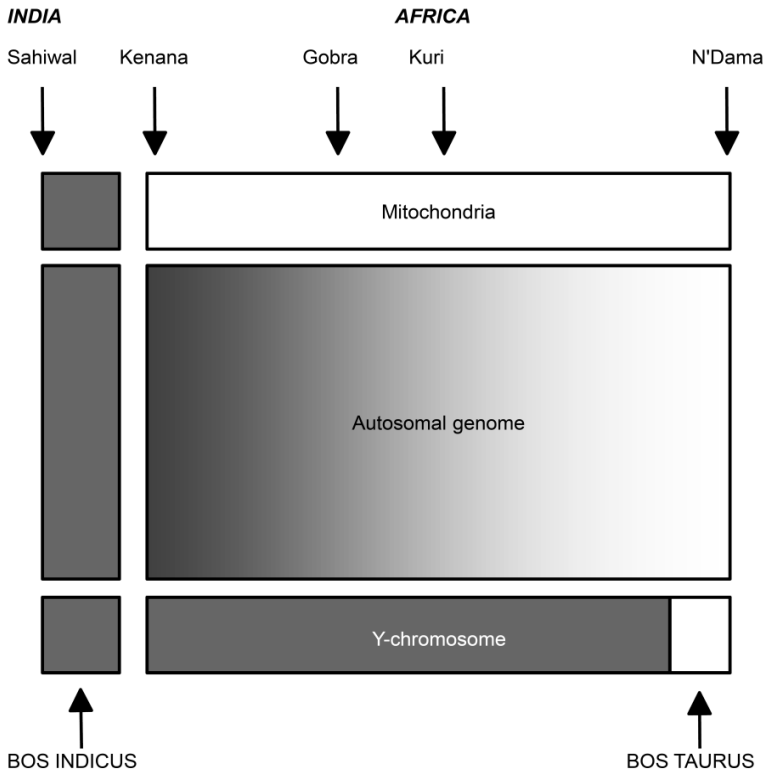

Figure 10. Zebu genomic introgression in African cattle as illustrated by three different genetic systems. Redrawn with permission from Bradley *et al.* (1998)

The New World, more specifically North and Central America welcomed its first cattle, which were taurine-natured, in the 15th century thanks to Christopher Colombus, whereas indicine cattle breeds were imported to Brazil and other countries in the 19th century (McTavish *et al.*, 2013).

3.2. Characteristics of zebu cattle morphology and adaptations to the environment

Zebu cattle are intermittently referred to as *Bos indicus* (MacHugh *et al.*, 1997; Hanotte *et al.*, 2000), *Bos primigenius indicus* (Ajmone-Marsan *et al.*, 2010; Hall *et al.*, 2013) or *Bos taurus indicus* (Cronin *et al.*, 2013). In official taxonomy, these cattle are defined as *Bos taurus indicus*, although the debate is still on-going after proof of different ancestry (ITIS, 2013).

Whatever their true name might be (in this work we will use "zebu" or "*Bos indicus*" from now on), they are the most prevalent type of cattle in the tropics (Figure 11) and are clearly distinguishable from *Bos taurus* cattle. Most strikingly, they have a thoracic hump and dewlap (Bradley *et al.*, 1998) (Figure 9). Often they have a smaller frame than their counterparts. Anatomically, they also differ from *Bos taurus* cattle in cranial angles (possibly indicating a slightly different way of holding their head) and because of the presence of a flat orbital rim (versus a sharp transition in *B. taurus*) (Grigson, 1980).

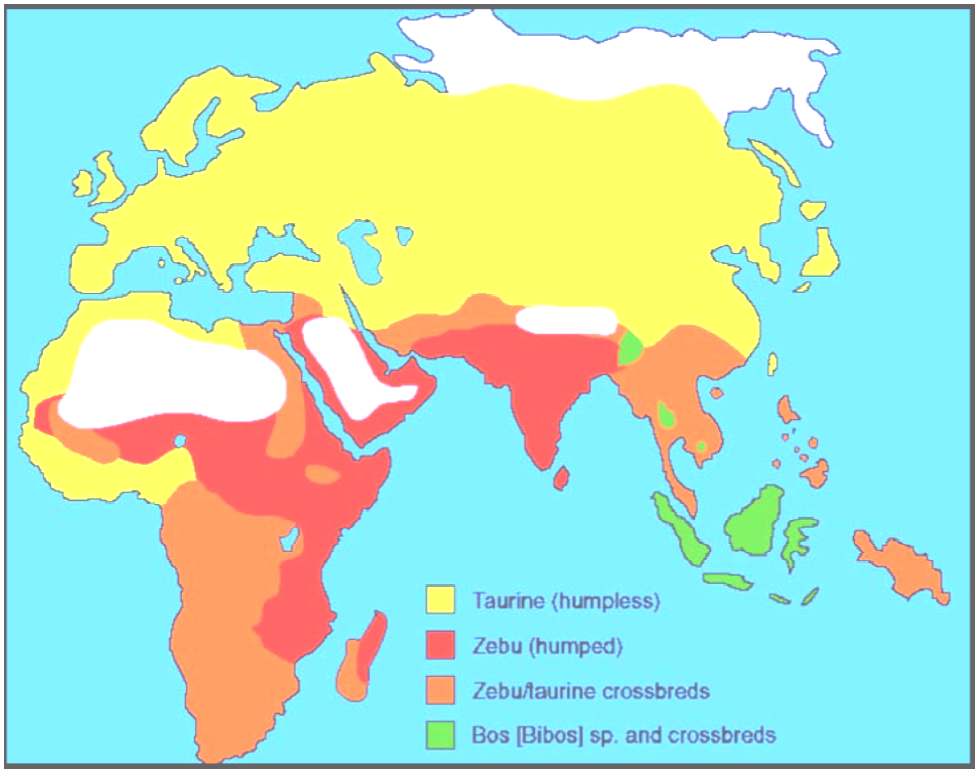

Figure 11. Distribution of different cattle types in the Old World (reproduced with permission from Caramelli (2006)). White zones represent areas with low numbers of cattle

Moreover, their main adaptive characteristic, heat tolerance, is related to their skin anatomy, with well-developed sweat and sebaceous glands and a smooth coat with primary hair follicles, they are able to evaporate more moisture and maintain normal body temperature in extreme environments. Furthermore, they have a lower respiration rate while kept under such conditions (Turner, 1980).

These cattle are also renowned for their ability to thrive on high roughage diets, making more efficient use of the low energy content (Turner, 1980) and digesting a roughage diet more quickly with higher production of rumen ammonia (Hunter & Siebert, 1985). Zebu cattle might even have adapted to the presence of tannins, an antinutritional compound, through the production of salivary proline-rich proteins, usually not seen in grazers (Yisehak *et al.*, 2011, 2012). Whether the latter are inherent to zebu cattle or are adaptive changes to grazing in this environment, is not known. Zebu cattle also seem to have a higher resistance towards rinderpest, ticks, nematodes and *Babesia* as well as *Brucella abortus* (Bradley *et al.*, 1998; Macedo *et al.*, 2013), but a lower resistance towards trypanosomes (MacHugh *et al.*, 1997).

When expressed in performance characteristics, zebu cattle are less excelling. They are slow growers (Turner, 1980) and their meat is less tender than in *B. taurus* cattle, due to reduced post-mortem degrading of myofibrillar proteins (Whipple *et al.*, 1990). Their puberty also starts much later and they have long periods of postpartum anoestrus, illustrated by a higher age at first calving (57 vs. 47 months) and long calving intervals (457 vs. 450 days, not significantly different) (Abeygunawardena & Dematawewa, 2004; Abraha *et al.*, 2009). It is also well known that they have a lower milk yield (2.7 vs. 4.5 l/day at beginning of lactation) and shorter lactation length (222 vs. 241 days) than crossbred cattle (Abraha *et al.*, 2009).

3.3. Trace elements in zebu cattle: what do we know?

3.3.1. Considerations on search methodology

It is difficult to keep track of zebu trace element research. Often, the terms zebu or *Bos indicus* or alikes are not really used and an educated guess needs to be used to define the type of cattle present at a certain study location.

Based on the maps of Bradley *et al.* (1998) and Caramelli (2006) (Figure 11), we presumed cattle were indicine when originating from India, whereas for African cattle, they were regarded as zebu if they were referred to as local, indigenous and if they originated from Chad, Sudan, Eritrea, Djibouti, Ethiopia (with exclusion of the taurine Sheko breed in the South-West), Somalia, Kenya, Tanzania, Mozambique, Malawi or Central African Republic.

Other African countries were only partially inhabitated with indicine cattle in addition to crossbreds or even taurine breeds (Bradley *et al.*, 1998; Caramelli, 2006), we, therefore, excluded these from our literature search. Furthermore, data from Yemen, Oman and the Emirates were added. Studies performed in Brazil were also included based on the presence of the indicine Nellore and Guzerat breeds (McTavish *et al.*, 2013).

Any other studies specifically stating the use of zebu cattle were also taken into account. Finally, an appropriate search term was built in order to identify all available research on trace elements in zebu cattle (Table 9) .

Table 9. Search terms used for literature review on trace elements in zebu cattle

Search engine and terms	Number of Results	Number of Relevant studies
Pubmed search	101	23
(zebu[tw] OR zebus[tw] OR zebu-influenced[tw] OR bos indicus[tw] OR bos taurus indicus[tw] OR bos primigenius indicus[tw] OR cattle[mesh] OR cattle[tw] OR bull[tw] OR bulls[tw] OR calve[tw] OR calves[tw] OR heifer[tw] OR heifers[tw] OR cow[tw] OR cows[tw]) AND (Brazil[Mesh] OR India[Mesh] OR Ethiopia[Mesh] OR Kenya[Mesh] OR Djibouti[Mesh] OR Somalia[Mesh] OR Eritrea[Mesh] OR Sudan[Mesh] OR Chad[Mesh] OR Tanzania[Mesh] OR Mozambique[Mesh] OR Malawi[Mesh] OR Central African Republic[Mesh] OR Yemen[Mesh] OR Oman[Mesh] OR United Arab Emirates[Mesh] OR Brazil[tw] OR India[tw] OR Ethiopia[tw] OR Kenya[tw] OR Djibouti[tw] OR Somalia[tw] OR Eritrea[tw] OR Sudan[tw] OR Chad[tw] OR Tanzania[tw] OR Mozambique[tw] OR Malawi[tw] OR Central African Republic[tw] OR Yemen[tw] OR Oman[tw] OR Emirates[tw]) AND (Trace Elements[Mesh] OR Sulfur[Mesh] OR Iron[Mesh] OR Cobalt[Mesh] OR Iodine[Mesh] OR Copper[Mesh] OR Selenium[Mesh] OR Zinc[Mesh] OR Manganese[Mesh] OR Molybdenum[Mesh] OR trace mineral[tw] OR micromineral[tw] OR trace element[tw] OR trace elements[tw] OR mineral[tw] OR minerals[tw] OR iodine[tw] OR copper[tw] OR selenium[tw] OR zinc[tw] OR manganese[tw] OR iron[tw] OR cobalt[tw] OR molybdenum[tw] OR sulfur[tw] OR sulphur[tw])		
Web of Knowledge search	328	50
(zebu OR zebus OR zebu-influenced OR bos indicus OR bos taurus indicus OR bos primigenius indicus OR cattle OR bull OR bulls OR calve OR calves OR heifer OR heifers OR cow OR cows) AND (Brazil OR India OR Ethiopia OR Kenya OR Djibouti OR Somalia OR Eritrea OR Sudan OR Chad OR Tanzania OR Mozambique OR Malawi OR Central African Republic OR Yemen OR Oman OR Emirates) AND ("trace mineral" OR micromineral OR "trace element" OR "trace elements" OR mineral OR minerals OR iodine OR copper OR selenium OR zinc OR manganese OR iron OR cobalt OR molybdenum OR sulfur OR sulphur)		
Total	429	58[a]

[a]The total number of relevant studies was calculated by subtracting any identical studies from the searches using the two search engines from the total number of relevant studies found.

3.3.2. Overview of the literature available

To date, research on trace element nutrition in zebu cattle still predominantly consists of reports on the evaluation of trace element supply through the *diet* and the trace element *status* of zebu cattle (Frøslie *et al.*, 1983a,b; Khalili *et al.*, 1993; Abdelrahman *et al.*, 1998; Gizachew *et al.*, 2002; Gowda *et al.*, 2004). Most often, an inadequate supply in Cu, Zn is seen together with very high levels of the Cu antagonist Fe, concomitantly reported with mainly severe shortages of Cu in cattle. However, not all elements were analysed in all reported studies, so data are incomplete.

Some studies did take the effort to look at the broader picture related to the trace element status in zebu cattle. They reported on the presence of the *soil-plant-animal flow* in zebu grazing lands (association of soil-plant-animal concentrations: Kumaresan & Bujarbaruah, 2010), tried to unravel factors related to the trace element content of soil and forages growing on this soil (parental material: Jumba *et al.*, 1995). Some authors called for a broad view on certain disorders, with minerals as an integrated part of the cure (postpartum anoestrus: Brar & Nanda, 2008). Another study searched for natural alternatives for mineral supplementation (Kabaija, 1989).

The effect of trace element status on zebu *anti-oxidant status* was not intensively studied before, but Sharma *et al.* (2005) did see an increase of SOD and Cp in Cu supplemented crossbred dairy cattle. When considering the effect on *immunity*, Dang *et al.* (2013) reported a better cellular immunity, namely higher phagocytic activity pre- and post-partum in Cu and Zn supplemented as well as a higher B lymphocyte proliferation pre- and post-partum in Cu supplemented zebu beef cattle, whereas Sharma *et al.* (2005) did not report a significantly higher phagocytic activity in Cu supplemented crossbred dairy cattle. Se supplementation did also cause a better humoral immunity, namely a higher antibody response in zebu beef cattle (Reis *et al.*, 2009). Mandal *et al.* (2007) on the other hand, observed a higher antibody response in Zn supplemented crossbred dairy cattle, but only with the organic Zn supplement. Studies on other trace elements are absent.

Focussing on the effect of trace elements on zebu *production*, Howard (1970) and Roeder (1980) observed a greater weight gain in zebu beef cows due to treatment with Cu and Co, respectively, while both elements were supplemented in the two studies. Kabaija & Little (1991) on the other hand, did not report any difference in weight gain caused by Cu (and P) supplementation in zebu beef bulls. Moreover, in crossbred dairy cows, Zn supplementation did not induce a higher milk yield (Sharma & Joshi, 2005), whereas the effect of the same element on nutrient digestibility and growth in crossbred dairy bulls was also absent (Mandal *et al.*, 2007).

Studies reporting on the trace element status in zebu cattle often include analysis of plasma as well as liver samples (Frøslie *et al.*, 1983a,b; Khalili *et al.*, 1993). However, only few studies report trace element concentrations in other *tissues*, and bodily fluids (milk:

Murray *et al.*, 1980; Salih *et al.*, 1987; Admasu *et al.*, 2008; Raghu, *in press*; semen: Aguiar *et al.*, 2012; meat: Giuffrida-Mendoza *et al.*, 2007; Oyaro *et al.*, 2007).

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Scientific Aims

Throughout the animal kingdom, trace elements are known to be essential for optimal health (Bender, 2007). Within the bovine species, numerous historical studies confirmed their importance, as supplementation of these elements alleviated elusive symptoms and improved cattle production (McDowell & Arthington, 2005).

From then on, worldwide, authors reported trace element shortages and overload in natural pastures, indicating an omnipresent risk for trace element imbalance in grazing cattle (Abdelrahman *et al.*, 1998; Mortimer *et al.*, 1999; Govasmark *et al.*, 2005a,b). Indeed, the grazing cow has its role in the trace element cycling through soil, plant and animal, and the bovine trace element status is directly influenced by this flow, although many influencing factors are yet to be unravelled (Suttle, 2010).

Furthermore, studies investigating the effects of trace elements on some body functions, such as immunity (Spears, 2000; Weiss & Spears, 2006; Spears & Weiss, 2008) as well as on performance (Ward & Spears, 1997; Engle & Spears, 2000) often produce conflicting results. Additionally, few studies investigated their impact under practical farming conditions (Wichtel, 2003). Finally, the influence of trace element status in edible tissues and milk during different stages of trace element supply, is not well understood (García-Vaquero *et al.*, 2011; Rey-Crespo *et al.*, 2013).

In tropical areas, several environmental characteristics (e. g. parent material, heavy rainfall) increase the risk for trace element imbalances in cattle (Dudal, 1980). Overall, in such regions, poor grasslands remain the primary bottleneck for optimal cattle production (McDowell & Arthington, 2005).

The zebu cattle type (*Bos indicus*), domesticated independently from *Bos taurus* cattle, is spread around the globe with a predominant presence in these tropics, e. g. South Asia and large parts of Africa (MacHugh *et al.*, 1997; Bradley *et al.*, 1998; Hanotte *et al.*, 2000). Zebu cattle are renowned for their adaptive traits to a harsh environment, with a high tolerance towards heat stress, high disease burden and poor dietary quality (Turner, 1980; Bradley *et al.*, 1998; Macedo *et al.*, 2013). However, few work was performed on trace elements in *Bos indicus* (-influenced) cattle.

In Ethiopia, the world's fifth largest cattle holder (FAO, 2013), poor communal grass lands are often characterised by erosion and overgrazing (Devi *et al.*, 2008). A coherent grazing management policy is absent and farmers have low financial resources to supplement zebu cattle with high quality grains or by-products, let alone commercial mineral supplements. In this country, beef oriented zebu cattle dominate the cattle population, in addition to dairy oriented crossbreeds with taurine Holstein-Friesians, a mix of sanga and zenga cattle (Dadi *et al.*, 2008) and the small indigenous taurine Sheko population (≤ 4000 individuals left according to Taye *et al.* (2009)).

The Gilgel Gibe catchment (Figure 1), SW Ethiopia, is a typical area in the country and was therefore selected as our study site to investigate several aspects of trace elements in zebu cattle. The area also forms the main subject of a multidisciplinary research project (IUC-JU programme of VLIR-UOS) investigating the impact of a hydroelectric power plant with adjacent reservoir and dam on human and animal health, ecology and agronomy, in order to improve the life quality of local communities.

In this area, farmers subside from mixed farming systems (Moti *et al.*, 2012) and cattle seem to be mainly of the indicine Guraghe-type (DAGRIS, 2007). Herds of such cattle are typically free-ranging on poor pastures. In and around Jimma, the largest city in the area, urban dairy farming is commonly practiced. Most cattle on these rather small farms are crosses of local zebu and Holstein Friesians. The animals are kept on a zero-grazing regime with a cut-and-carry feeding system for forages, fed in combination with concentrates and by-products (Belay *et al.*, 2012).

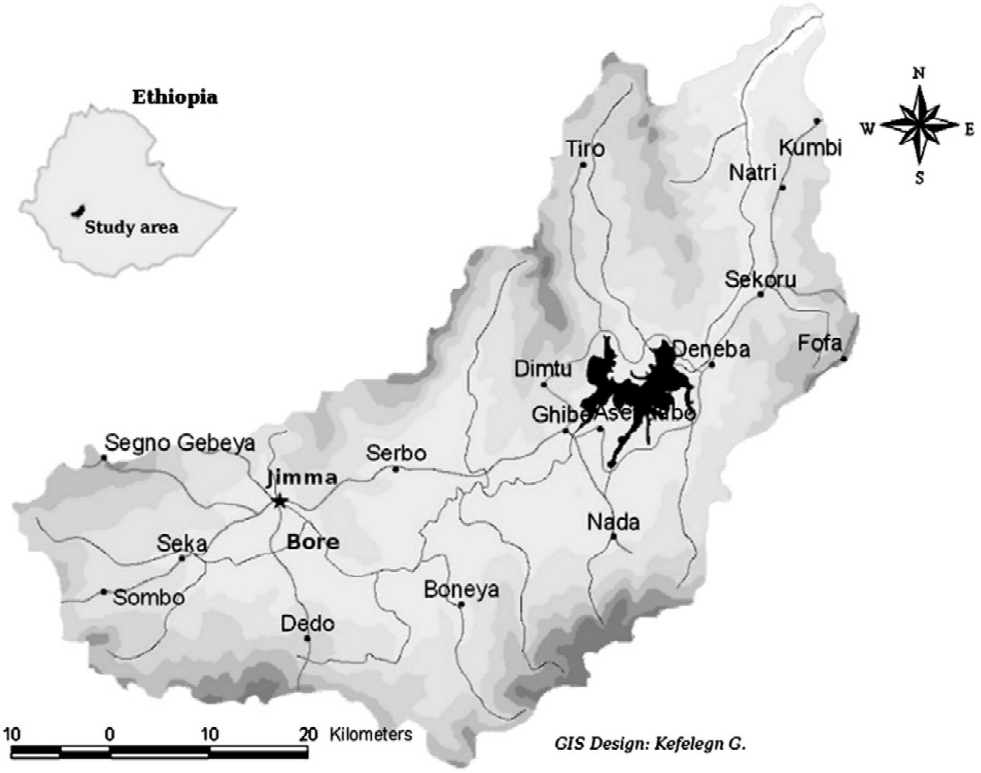

Figure 1. The Gilgel Gibe catchment in Ethiopia
(reproduced with permission from Van Ranst *et al.* (2011))

In this doctoral thesis, our objective was to obtain a broad insight on trace element nutrition in zebu cattle, while compiling research questions arisen from paragraphs above. In this respect, the zebu cattle type formed both tool and subject of this thesis, as studying trace elements in zebu cattle, would increase overall knowledge on trace elements in the bovine species.

More specifically, as such, our objectives were to:

-**Evaluate the trace element *supply* for zebu (-influenced) cattle**, through the compilation of data from an exemplary tropical region with literature information.

-**Evaluate the trace element *status* in zebu (-influenced) cattle**, again in an exemplary tropical region as compared to literature. Also, it also was our intention to compare different sampling methods to evaluate this trace elements status and to investigate the use of new samples.

-Gain more insights on factors influencing the soil-plant-animal flow. In particular, we wanted to explore the influence of the environment and management as well as the effect of plant factors, such as plant type. Additionally, our objective was to fully study the impact of animal factors, including differences with *Bos taurus* cattle.

-Investigate the effect of status in beef and dairy zebu (-influenced) cattle on different aspects of bovine health and production. We were interested in identifying effects of trace element status on bovine anti-oxidant status, immunity and disease resistance in addition to the impact on production, both in beef and in dairy cattle. Moreover, we aimed to evaluate trace element status effects on trace element storage in edible tissues abd trace element secretion in milk, and to analyse their potential for human nutrition

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Research Chapters

1

Ingested plant species, environmental factors and grazing management affect estimated dietary trace element concentrations in grazing zebu (*Bos indicus*) cattle

Adapted from:

V. Dermauw, T. Van Hecke, K. Yisehak, L. Duchateau, G. Du Laing, E. Van Ranst, G. P. J. Janssens. To be submitted.

As in many tropical countries, natural pastures are the main source of nutrients for cattle in Ethiopia. In this study, we evaluated trace element concentrations in ingested plant species, estimated the total dietary (ETD) trace element content and investigated the effect of environment and management in nineteen herds of zebu cattle (Bos indicus) grazing at the Gilgel Gibe valley, Ethiopia. Ingested plants were analysed for Cu, Co, Fe, Mo, Mn, S, Se and Zn and total dietary intake estimated through ten minute interval bite observation. Inadequate Cu concentrations were present in 72% of samples, whereas Se and Zn shortages were present in 59 and 43% of plants, respectively. In general, ETD concentrations mirrored this, and contained disadvantageous Cu:Mo (19%) and Fe:Cu (41 %) ratios but diets were not considered Zn deficient. Concentrations of Cu and S were higher in herbaceous and woody plants than in grasses and crop resides, whereas Co concentrations were higher in herbaceous than in other plant types. Differences between plant types were also present for Fe. Both plant Mn and Zn concentrations were higher at low versus medium altitude. Plants growing on Nitisol-Acrisol-Ferralsol soils contained higher amounts of Fe than on Planosol-Vertisol (PV) soils. Lower ETD concentrations of Cu and Fe and higher Mo concentrations were present in herds grazing on PV soils. Communal grazing herds ingested diets with higher ETD Mo and Mn concentrations. Herding distance positively affected ETD Mn concentrations. Finally, supplementing cattle diets with crop residues had a negative effect on ETD Zn and Se concentrations. Overall, micro mineral deficiency is very likely to develop in cattle ranging at the studied area. This study is pointing to the major impact of environment and management, closely related, on the trace element supply for grazing herds.

1.1 Introduction

Cattle production in tropical areas is predominantly extensive and smallholders are majorly dependent on natural pastures and rangelands to provide their cattle with a satisfactory nutrient supply (Leng, 1990). Such pastures are often poor in trace elements (McDowell & Arthington, 2005). In East Africa, earlier studies described low levels of Se, Cu and Zn in native pastures (**Chapter 4**; Faye & Grillet, 1984; Gizachew *et al.*, 2002; Kabaija & Little, 1987; Khalili *et al.*, 1993). Not surprisingly, trace element deficiences (f. e. Cu, Se, I) are very common in grazing cattle in this area (**Chapter 2, 4, 5 & 6**; Schillhorn van Veen & Loeffler, 1990).

It is well known that minerals in particular are subjected to a soil-plant-animal continuum (Gupta *et al.*, 2008; Reid & Horvath, 1980), with influencing factors at all levels. Jumba *et al.*, (1995) reported on the effect of geology and elevation on herbage mineral concentrations. The effect of grazing management is less studied. Coppolillo (2000) did describe the effect of ranging management practices on productivity in pastoral herds in Tanzania. Grazing in larger herds for longer distances accounted for a loss in milk yield and BCS.

Our objective was to gain more insights on the factors affecting the flow of trace elements from soil through plant to the animal in tropical grazing cattle. More specifically, we explored 1) the effect of plant type and environment on trace element concentrations of ingested plants as well as 2) the effect of environment and management practices on total dietary trace element concentrations of grazing zebu (*Bos indicus*) cattle.

1.2 Materials and methods

1.2.1. Study area, herd selection and environment

An area within a radius of 35 km around the town of Jimma, located in the Gilgel Gibe catchment, South-West Ethiopia, was selected as our study site. The Gilgel Gibe catchment has an elevation range of 1096-3259 m above sea level (asl) and a subhumid climate. The main rainy season, *kiremt,* presents itself between May and September. This study was executed at the end of the *kiremt*, from September to October. Lower valley areas receive a mean annual rainfall of 1300mm, whereas upland regions around 2000mm. Mean minimum, maximum and average temperatures recorded at the Jimma station (1800m altitude) are 11 °C, 25 °C and 17 °C, respectively (Van Ranst *et al.*, 2011). Smallholders typically let their cattle roam free on communal rangelands (Yisehak *et al.*, 2012).

The study area was divided in three subregions, according to elevation, a "low" region within 1700-1800m asl, an "intermediate" region between 1800-2000m asl, and a "high" region between 2000 and 2200 m asl. Randomly, 19 herds of free ranging zebu (*Bos indicus*) cattle were selected over these three regions and observed during one grazing day (Figure 1.1.).

The soil types found in the study area were identified based upon visual inspection of the top layer. The most commonly found World Reference Soil (WRB) soil groups (IUSS Working Group WRB, 2007) at the Gilgel Gibe catchment are Nitisols, Planosols and Vertisols (Van Ranst *et al.*, 2011). The free-draining Nitisols, with some minor inclusions of Ferralsols and Acrisols (soil association NAF) occur in the level to hilly uplands. These finely textured (clayey) weathering products of volcanic rocks are dominantly dark reddish brown to yellowish red in color. The soilscapes, with associations of Planosols and Vertisols (PV) occur in the flat, lower river terraces and valley areas filled up with sediments. Planosols in this catchment have an abrupt textural change at about a depth of 40 cm separating a bleached, (dark) gray to light gray, silty topsoil from a black, heavy clayey vertic horizon (Vertisol) (IUSS Working Group WRB, 2007).

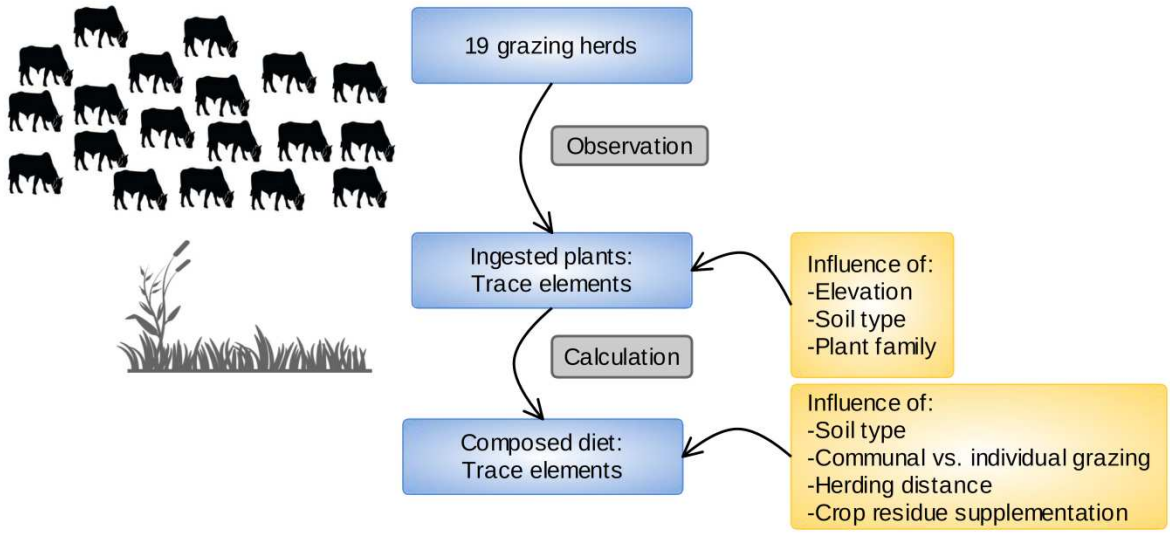

Figure 1.1. Experimental design employed to investigate the impact of environment, plant and management on trace element supply in zebu herds (n = 19) grazing at the Gilgel Gibe catchment

1.2.2. Management

Management practices were evaluated based upon grazing strategy, herd size, supplementation with crop residues, and the total distance/herding radius covered. The grazing strategy (GS) was either communal, in which different herds shared communal grazing lands, or individual, in which small herds belonging to individual farmers grazed on separate pastures. The crop residue (CR) supplementation was expressed as % of the total observations (see section 1.2.3.). The herding distance (HD) was the total distance covered from and travelling back to the home stable during the observation day, measured using a global positioning system (Garmin® eTrex Legend HCx, Garmin International, Olathe, Kansas), attached to the herd observers.

1.2.3. Ingested plant species and estimation of total dietary trace element concentrations

In every herd, one adult cow was closely monitored during the daily grazing period, from the moment the herd left the stable until it returned in the evening. Every 10 minutes, ingested plant species were recorded and a sample of all ingested plants was

collected. The total number of ingested plant species was also recorded. Plants were classified as one of the following plant types: grasses, crop residues, herbaceous plants or woody plants. Grasses (G) included the "true grasses" or Poaceae and the "sedges" or the Cyperaceae. Crop residues (CR) were defined as stovers from cultivated plant species offered by farmers to their cattle. Herbaceous plants (H) contained sampled forbs whereas the group of woody plants (W) contained leaf samples from trees and shrubs. One plant observation was equal to 1 point, several species recorded during an observation point were allocated 1 point divided by the number of species recorded, thereby estimating the proportion of a single ingested plant species in the total diet. To obtain the estimated total dietary (ETD) trace element concentrations, the proportion of each ingested plant was multiplied by the trace element contents of the plant and all of them were summed. If no sample could be obtained of some plants due to its small size, the mineral concentration of the known percentage of ingested plants was extrapolated to 100%. Although earlier reported bite masses (Stobbs (1973) 0.29-0.44 mg OM/bite; (Gibb *et al.*, 1999) 0.07-0.20 mg OM/bite) could theoretically allow us to calculate accurate trace element intake in herds, reported ranges are quite variable, and refer to bite masses on pastures. No data exist for crop residues and woody plants. We did, therefore, not correct for DM content or bite mass of ingested plants.

1.2.4. Mineral analyses

Plant samples were oven dried at 65 °C for 72 hours and ground through a 2 mm screen. All plant samples were ashed through microwave destruction with 10 ml HNO_3 (Ultrapure analytical grade for trace element analysis) in closed vessels followed by filtration and analysis for concentrations of Cu, Fe, Mo, Zn, Mn, Se and S by means of ICP-OES and ICP-MS (Elan DRC-e, Perkin Elmer, Zaventem, Belgium). All glassware and microwave vessels were pre-rinsed with diluted HNO_3. Throughout the mineral analyses, a quality control program was applied. Trace element recovery rates from certified reference material (Rye grass ERM-CD281, BCR Reference Materials, Belgium) were measured. Average recovery was 97%, with a range between 86% (Zn) and 107% (Mo). Detection limits in acid digest were determined as: Mn 0.35 µg/l, Cu 0.25 µg/l, Mo

0.33 µg/l, Se 0.13 µg/l, Fe 21.4 µg/l, Zn 16.4 µg/l and Co 0.14 µg/l. Alongside samples, standards were run frequently. Furthermore, all analytical results were blank-corrected.

1.2.5. Statistical analyses

Statistical analysis was performed using SAS Version 9.3 (SAS Institute Inc., Cary, NC). Concentrations of plant trace elements were not normally distributed and therefore log transformed. A multivariable fixed effects model with elevation region, soil type and plant group as categorical fixed effect was fitted to the log of the plant trace elements. Geometric means (GM), and geometric standard error of the means (GSEM), which represent the antilog of the arithmic mean and arithmic standard error of the means, respectively, of the log-transformed data, were given here. To determine the association between categorical variables (i. e. management and environment factors), a Fisher's Exact test was used. To determine the effect of the physical environment and management on ETD trace element concentrations, a fixed effects model with one categorical or one continuous covariate at a time was fitted to the different response variables. Significance was declared at a probability level of $p < 0.050$.

1.3 Results

1.3.1. Ingested plant species and influence of plant type and environment

Sampled plant species (n = 58) on the two soil types are shown in Table 1.1. Only 28 % of plant samples contained adequate Cu concentrations (NRC, 2000). Many plants contained toxic levels of Fe (31%), some of Mo (2%) and S (2%) (NRC, 2005). Furthermore, deficiencies of Se and Zn were found in 59% and 43% of samples, respectively, whereas concentrations of Co and Mn seemed to be adequate in sampled plants (NRC, 2000) (see **Introduction**: Table 3).

Table 1.1. Sampled plant species based upon observed ingestion by grazing cattle around the Gilgel Gibe catchment in South-West Ethiopia.

	Plant type			
Soil	Grasses	Crop residues	Herbaceous	Woody plants
NAF	*Andropogon abyssinicus*	*Ensete ventricosum*	*Aspilia africana*	*Coffea arabica*
	Brachiaria spp.	*Musa* spp.	*Aspilia* spp.	*Erythrina brucei*
	Cynodon spp.	*Pisum sativum*	*Bidens macroptera*	*Grewia ferruginea*
	Cyperus spp.	*Saccharum officinarum*	*Bidens pilosa*	*Maytenus obscura*
	Melinis spp.	*Sorghum bicolour*	*Centella asiatica*	*Persea americana*
	Panicium coloratum	*Zea mays*	*Desmodium uncinatum*	*Physalis peruviana*
	Pennisetum clandestinum		*Indigofera* spp.	*Premna schimperi*
	Pennisetum sphacelatum		*Ipomoea batatas*	*Rhus glutinosa*
	Pennisetum thunbergii		*Persicaria nepalensis*	*Sida rhombifolia*
			Satureja paradoxa	*Sida* spp.
			Satureja spp.	*Vernonia adoensis*
			Trifolium spp.	*Vernonia amygdalina*
PV	*Cynodon* spp.	*Sorghum halepense*	*Aspilia mossambicensis*	*Eucalyptus camaldulensis*
	Cyperus spp.		*Centella asiatica*	*Psidium guajava*
	Hyparrhenia hirta		*Hygrophila auriculata*	
			Senna didymobotrya	
			Sesbania sesbon	

NAF = Nitisol, Acrisol, Ferralsol associations, PV = Planosol, Vertisol assocations

Differences in mineral concentrations (shown as geometric means) according to plant type are presented in Table 1.2. Briefly, herbaceous and woody plants contained higher Cu and S concentrations than grasses and crop residues ($p < 0.001$). Co concentrations were higher in herbaceous plants than in other types of plants ($p = 0.005$). For Fe, differences between plant types existed, but they were not clear cut ($p < 0.001$).

Table 1.2. Trace element concentrations (GM)(in mg/kg DM, S in % DM) in different plant types sampled around the Gilgel Gibe catchment in South-West Ethiopia.

	Plant type					
Mineral	Grasses (n = 20)	Crop residues (n = 7)	Herbaceous plants (n = 18)	Woody plants (n = 13)	GSEM	p-value
Co	0.48[a]	0.25[a]	0.92[b]	0.35[a]	1.128	0.005
Cu	5.4[a]	4.2[a]	8.4[b]	10.9[b]	1.072	<0.001
Fe	769[ab]	312[ac]	1276[b]	286[c]	1.151	<0.001
Mn	166	161	222	185	1.111	0.952
Mo	0.85	0.56	0.93	0.34	1.147	0.122
S	0.15[a]	0.13[a]	0.25[b]	0.24[b]	1.066	<0.001
Se	102	74	116	74	1.091	0.340
Zn	33	17	38	34.6	1.078	0.082

[a,b]Different letters within a row differ significantly ($p < 0.050$), GM = geometric mean, GSEM = geometric standard error of the mean.

Furthermore, higher Mn concentrations were present in plants sampled at low elevation than at medium and high elevation (366 versus 157 and 113 mg/kg DM (GM) ± 1.11 (GSEM), $p = 0.011$). Zinc concentrations were lower at medium elevation than at lowest elevation (23.5 versus 41.0 mg/kg DM ± 1.15, $p = 0.020$). Plant sampled on NAF soils contained slightly more Fe than on PV soils (597 vs. 586 mg/kg DM ± 1.15, $p = 0.048$). No other significant differences in trace element concentrations according to elevation or soil type were present.

1.3.2. Association between environment and management

Soil type (Figure 1.2) and elevation were significantly associated ($p < 0.001$), with the typical occurrence of Planosol/Vertisol associations (PV) at lower elevation. Soil type

and elevation were also significantly associated with herd type, as more communal grazing practices were used at lower elevation and PV soils (p = 0.010, p = 0.030).

The herding distance was affected by soil and herding type, with cattle grazing for longer distance on PV soils (4.1 vs. 1.9 ± 0.5 km, p = 0.020), and in communal herds (4.9 vs. 1.7 ± 0.5 km, p = 0.001) than on NAF soils. On the contrary, the amount of crop residues provided to the grazing cattle, was not affected by soil or herding type (both p > 0.050) (data not shown).

Figure 1.2. Differences in grazing environment of observed herds (n = 19) at the Gilgel Gibe catchment, Ethiopia. **Left**: greyish waterlogged Planosol/Vertisol soil type, **Right**: red well-drained Nitisol/Acrisol/Ferralsol soil type.

1.3.3. Estimated total diet and influence of environment and management

ETD trace element concentrations are presented in Table 1.3. All composed diets were Cu deficient (19/19 herds), some were Se (4/19) or Zn (1/19) deficient, upon comparison with requirements for *Bos taurus* beef cattle (NRC, 2000). A few diets (3/19) had a Cu:Mo ratio considered to indicate a risk for Mo antagonism in combination with S concentrations > 0.15 % kg DM, whereas the Fe:Cu ratio was too high in all diets (19/19) (Suttle, 2010).

We then considered the impact of environment on trace element supply. Due to the high association between soil type and elevation, we mainly focussed on the effect of soil type. Soils of the PV type had lower number of ingested plant species (10.7 vs. 17.2 ±

1.3), and grazing on these soils resulted in lower ETD Cu (4.7 vs. 6.4 ± 0.3 mg/kg DM) and Fe concentrations (765 vs. 1672 ± 183 mg/kg DM) whereas concentrations of Mo (1.47 vs. 0.96 ± 0.10 mg/kg DM) and Mn (331 vs. 173 ± 22 mg/kg DM) were lower on NAF soils (all $p < 0.050$). ETD concentrations of S tended to be lower on PV soils (0.15 vs. 0.19 ± 0.01 % DM, $p = 0.082$).

Table 1.3. Estimated total dietary mineral concentrations based upon observed ingestion in grazing zebu (*Bos indicus*) cattle herds (n = 19) at the Gilgel Gibe catchment, Ethiopia

Mineral	Mean		SD	Range			Recommendations[1,2]
Co, mg/kg DM	0.95	±	0.78	0.17	-	3.6	>0.10
Cu, mg/kg DM	5.8	±	1.3	4.1	-	8.0	>10
Mn, mg/kg DM	231	±	96	70	-	412	>20
Se, µg/kg DM	136	±	37	57	-	184	>100
Zn, mg/kg DM	41	±	7.0	26	-	51	>30
Mo, mg/kg DM	1.2	±	0.4	0.5	-	2.0	-
Fe, mg/kg DM	1338	±	798	400	-	3855	>50
S, % kg DM	0.17	±	0.04	0.13	-	0.27	>0.15
Cu:Mo ratio	6.0	±	3.1	2.4	-	12	>1.0-3.0
Fe:Cu ratio	222	±	96	98	-	510	<50-100

SD = standard deviation
[1,2]NRC (2000), Suttle (2010)

Within the influence of management, communal grazing herds had higher ETD concentrations of Mo and Mn (Mo: 1.4 vs. 1.0 ± 0.1 mg/kg DM; Mn: 185 vs. 329 ± 22 mg/kg DM) whereas the Cu:Mo ratio was higher in individually grazing herds (6.8 vs. 4.0 ± 0.7 mg/kg DM) (all $p < 0.050$). Herding distance positively impacted ETD Mn concentrations ($p = 0.030$) and tended to negatively affect the ETD Cu:Mo ratio ($p = 0.082$). Furthermore, the amounts of crop residues offered to grazing animals had a significantly negative effect on ETD Zn and Se concentrations ($p = 0.033$; $p < 0.001$ respectively) and tended to negatively affect ETD Mo and S concentrations ($p = 0.064$; $p = 0.063$ respectively).

1.4 Discussion

In our study, we observed 19 grazing cattle herds in the Gilgel Gibe valley, Ethiopia, in order to obtain information about trace element content of ingested plant species, to estimate total dietary trace element concentrations and to evaluate the effect of management and environment.

Trace element analysis indicated that the available and ingested vegetation in the area is most likely to induce trace element deficiencies in ranging cattle. In general, the vast majority of plant samples as well as diets (expresseb as ETD), contained Cu concentrations considered deficient upon comparison with requirements for beef cattle (*Bos taurus*) as stated by NRC (2000). As the Cu absorption coefficient (A_{Cu}) in fresh herbage (1.4-2.5 %) is already lower compared to silages (4.9 %) or hays (7.3 %) (Suttle, 2010), these concentrations are detrimental for the Cu status of grazing cattle. Low Cu levels in forages in Ethiopia were earlier described (**Chapter 4, 5, 6 & 7**; Faye & Grillet, 1984; Gizachew *et al.*, 2002; Kabaija & Little, 1987; Khalili *et al.*, 1993). Not surprisingly, earlier research (**Chapter 2, 4, 5 & 6**) also found Cu deprived cattle grazing in the same region, based upon plasma and liver analyses.

The primary Cu shortage was probably aggravated by the high ETD Fe:Cu ratios and the high prevalence of even toxic plant concentrations. ETD Fe concentrations between 250 and 500 mg/kg can already cause Cu depletion in cattle (Bremner *et al.*, 1987; Phillippo *et al.*, 1987). High concentrations of other antagonists Mo and S can worsen this situation through the formation of CuS and/or thiomolybdates in the rumen. Thiomolybdates can either bind Cu and form an insoluble complex or can be absorbed, tightly binding Cu to plasma albumin (Gooneratne *et al.*, 1989). Based upon ETD S concentrations and Cu:Mo ratios, in at least some herds, a risk for Mo and S antagonism was present. Even higher concentrations of Mo were found in Ethiopian pastures by **Chapter 4** and Faye & Grillet (1984), whereas higher S concentrations were reported by **Chapter 4**.

Furthermore, deficiencies of Se and Zn in plant samples were prominent, but for Zn, this was not reflected in low ETD Zn concentrations. Earlier, Faye & Grillet (1984), Gizachew

et al. (2002) and Khalili *et al.* (1993) found rather low plant Zn levels whereas adequate ranges were reported in **Chapter 4**. Furthermore, our low Se values agree with low Se concentrations in grasses (**Chapter 4**) and with the findings in **Chapter 2** which observed marginally deficient Se content in 92% of sampled livers from cattle in the same valley. Manganese and Co concentrations were in the adequate range and consequently no deficiencies in cattle are expected for these elements. These results are in accordance with McDowell & Arthington (2005) who perceived Mn deficiencies to be very rare and shortage of Co exceptional.

Trace element concentrations differed significantly between plant types. In general, herbaceous plants contained the highest trace element concentrations, whereas distribution between other plant types was rather heterogeneous. Suttle (2010) does mention that legumineous plants often contain higher amounts of trace elements than pure grasses. Leafs from herbaceous plant species might be interesting candidates to use as a natural trace element supplement. However, caution is warranted since the highest Fe, Mo and S concentrations, as mentioned above, classic Cu antagonists, were also found in this plant type. Furthermore, amounts of minerals in woody plants might be unavailable due to the high amount of tannins potentially complexing these minerals (Yisehak *et al.*, 2012).

In the current study, there was a strong association between elevation and soil type. Elevation seemed to affect plant concentrations of Mn and Zn, which lowered with increasing elevation. Jumba *et al.* (1995) earlier reported lower Se and Cu concentrations at low elevation on volcanic bedrock whereas Gizachew *et al.* (2002) reported numerically higher concentrations of a range of macro- and microminerals on low elevation in Nitisol association areas. Considering the strong association of soil type and elevation in our study as well as geology differences in herbage trace element concentrations reported by Jumba *et al.* (1995), we doubt that a pure elevation effect, irrespective of soil type, does exist. The lower ETD Cu concentrations on PV-grounds might be explained by the reduction of Cu transport by mycorrhizal fungi associated with plant roots, in organic rich wetlands associated with PV (Ragnarsdottir & Hawkins, 2006). Gizachew *et al.* (2002) also mentioned that lowlands contain high amounts of organic matter. Moreover, in organic rich wetland soil precipitation of Cu sulfides may

occur, rendering Cu less mobile and less available to plants. Furthermore, the high molecular weight organic matter compounds in the solid soil phase may also reduce the availability of Cu (Du Laing *et al.*, 2009). Fox & Doner (2003) reported that at the same wetlands, Mo often accumulates until toxic concentrations, which can add to the effect of Cu deficient concentrations in the diets of cattle grazing on wetlands and is in line with our results.

As mentioned above, in general, PV soils were frequently present at lower elevation. On these soils, large communal herds were grazing for longer distances, the latter in accordance to earlier work by Coppolillo (2000). According to the same author, large herds tend to move more quickly through a field, leaving less time to ingest plants, and if this energy cost is not compensated by improved pasture availability, it is associated with a lower BCS. In our study, herding distance positively impacted Mn concentrations, but we did not detect a herding distance influence on ETD concentrations of other trace elements.

Finally, supplementation with crop residues did not prove to be beneficial for grazing herd trace element supply. According to McDowell (1988), crop residues provide up to 30 to 90 % of livestock feeds in Africa. Despite their nutritional potential, they generally also contain high concentrations of indigestible fibre (McDowell, 1988) and tannins (Mueller-Harvey *et al.*, 1988). The latter are capable of forming insoluble complexes with microminerals (Karamać, 2009) and high concentrations are associated with reduced Fe (Gillooly *et al.*, 1983) and Cu status in cattle (Yisehak *et al.*, 2012).

Conclusion

Zebu cattle grazing in the study area were under high risk for Cu deficiency, potentially aggravated due to Fe, Mo and S overload. Our data point to the relationship between plant mineral concentrations and plant type, soil and elevation. Furthermore, both soil type and management practices, intimately related, affected the estimated total dietary trace element concentrations.

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mineral response to dietary condensed tannins in free-ranging zebu cattle (*Bos indicus*) as a marker of habitat degradation. *Livestock Science*, 144, 275–280.

2

Mineral deficiency status of ranging zebu (*Bos indicus*) cattle around the Gilgel Gibe catchment, Ethiopia

Adapted from:

V. Dermauw, K. Yisehak, D. Belay, T. Van Hecke, G. Du Laing, L. Duchateau, G. P. J. Janssens (2013). Mineral deficiency status of ranging zebu (*Bos indicus*) cattle around the Gilgel Gibe catchment, Ethiopia. Tropical Animal Health and Production **45**, 1139-1147.

Mineral deficiencies in cattle, widespread in East Africa, impair optimal health and production and consequently, place a great burden on the farmers' income. Therefore, detection of shortages and imbalances of specific minerals is essential. Our objective was to evaluate the mineral status of grazing cattle around the Gilgel Gibe catchment in Ethiopia and associated factors. In study I, individual animal plasma and herd faecal Ca, P, Mg, Na, K, S, Fe, Zn, Mn and Cu concentrations were determined in adult zebu cattle (Bos indicus; n = 90) grazing at three altitudes around the catchment, whilst recording body condition score and sex. In study II, liver samples of adult male zebu cattle (n= 53) were analysed for Cu, Zn, Fe, Se and Mo concentrations and inspected for parasitic infections. Plasma and liver analyses revealed a Cu deficiency problem in the area, since 68 % and 47 % of cattle, respectively, were Cu deprived according to diagnostic criteria for Bos taurus cattle. High hepatic Mo concentrations in 17% of cases might reflect excessive dietary Mo intake. Liver Se and plasma Na concentrations were too low in 92 % and 80 % of cattle. Plasma Mn concentrations were largely below the detection limit. Plasma Cu as well as Ca concentrations were lower in the lowest compared to the highest altitude group ($p < 0.050$), whereas lean to medium cattle had lower plasma Cu concentrations ($p < 0.050$). No differences in hepatic mineral concentrations were detected between cattle with different types of parasitic infection. In conclusion, bovine mineral deficiencies were present in the Gilgel Gibe area and were associated with grazing altitude and body condition score.

2.1 Introduction

In Ethiopia, livestock are of major importance as a source of nutrition, employment and financial security (Halderman, 2003). However, farmers of zebu (*Bos indicus*) cattle face major constrains in providing optimal nutrition for their grazing herds. One of the problems frequently associated with cattle nutrition in the tropics is mineral shortage (McDowell & Arthington, 2005). For example, in East African cattle, studies report Cu (Ethiopia: Gizachew *et al.*, 2002), Zn (Sudan: Ahmed *et al.*, 2002), I (Sudan: Schillhorn van Veen & Loeffler, 1990) and Se deficiencies (Tanzania: Mtui *et al.*, 2007). Common disorders linked with mineral deprivation in grazing cattle in the tropics are, among others, fragile bones (P), pica and general poor productivity (Na), severe diarrhoea (Cu) and parakeratosis (Zn) (McDowell & Arthington, 2005). Hence, mineral deficiencies can compromise cattle health, and consequently, the income to the farmers. The aim of this study was to investigate the mineral status of grazing cattle around the Gilgel Gibe catchment in Ethiopia and to identify factors associated with this status. A survey was performed in which plasma and faeces were sampled in cattle herds grazing in the area in order to evaluate their mineral status. The association between this mineral status and grazing altitude, sex and body condition was investigated. A second study focussed on the trace element status of liver samples, obtained from the local abattoir, of cattle grazing around the Gilgel Gibe catchment in Ethiopia and the effect of parasitic infections on hepatic mineral concentrations.

2.2 Materials and methods

2.2.1. Animals and samples

Study I

At the Gilgel Gibe catchment, situated in south-western Ethiopia, a screening was conducted at the beginning of the Ethiopian spring season, called *tsedey* (October-

November). As in the rest of Ethiopia, cattle, owned by smallholders, are typically free-ranging on communal lands.

The catchment area was divided into three subregions, i.e. at low altitude ('1') adjacent to the dam (< 1700 m above sea level (asl), at intermediate altitude ('2') (1700-1900 m asl) and finally, at high altitude ('3') (> 1900 m asl). In every subregion, 6 herds were randomly included in the study, while in every herd, five adult Ethiopian highland zebu cattle (*B. indicus*) were randomly selected (n = 90). Sex and body condition score (BCS) of every animal were noted, the latter using the 1 to 9 point scale for zebu cattle as formulated by Nicholson & Butterworth (1986). BCSs were grouped into categories: scores 1, 2 and 3 as "lean", scores 4, 5 and 6 as "medium" and scores 7, 8 and 9 as "fat". A fresh faecal sample was rectally obtained from the animals and pooled per herd (Figure 2.1). A blood sample was taken from the vena jugularis using 20 G needles (MN-2038M) and sodium heparin tubes (VT-100SH, both Venoject®, Terumo, Leuven, Belgium) (Figure 2.1). After sampling, the blood tubes were immediately placed on iced water and within 2 hours after sampling, plasma was separated by centrifugation at 1500 × *g* for 10 minutes. All samples were stored at -20°C until analysis.

Figure 2.1. Sampling of zebu cattle grazing (n = 90) at the Gilgel Gibe catchment, Ethiopia. **Left:** faeces sampling. **Right:** blood sampling.

Study II

A survey was conducted at the municipal abattoir in Jimma, the largest city in the Gilgel Gibe catchment area, again at the beginning of the Ethiopian spring season (October). Fifty-three adult, Ethiopian highland zebu (*B. indicus*) bulls were randomly sampled post

mortem. Approximately 50 g of liver tissue was collected per animal. The presence of parasitic infections in the whole livers was determined by visual inspection and noted.

2.2.2. Chemical analyses

Plasma samples were subjected to destruction for mineral analysis with 5ml HNO_3 and, in between consecutive heating steps, with 3 ml H_2O_2. Faecal samples were oven-dried at 65° C for 72 hours, ground through a 2 mm screen and prepared for mineral analysis through dry ashing at 500 °C for 4 hours, followed by wet destruction with 10 ml HCl during consecutive heating steps. Afterwards, plasma and faecal samples were filtered and analysed for concentrations of Ca, P, Na, K, Mg, S, Fe, Cu, Zn and Mn by inductively coupled plasma optical emission spectrometry (ICP-OES) (Iris Intrepid II XSP with dual-view (axial and radial), Thermo Fisher Scientific, Aalst, Belgium). Liver samples were oven-dried at 65° C for 72 hours, ground through a 2 mm screen and were prepared for mineral analysis through microwave destruction with 3 ml HNO_3 and 3 ml H_2O_2 in open vessels followed by filtration. Hepatic Zn, Cu and Fe concentrations were analysed with ICP-OES (Vista MPX radial, Varian, Palo Alto, USA), while hepatic Se and Mo concentrations by means of inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, Perkin Elmer, Sunnyvale, CA, USA). Hepatic trace element concentrations were expressed on dry weight basis (DW).

2.2.3. Statistical analyses

All statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL). To enable analysis of variance, non-detectable mineral concentrations were assigned a value of half the detection limit. Normal distribution of data was then evaluated with box-plot graphs. In *study I*, all data were submitted to a mixed model with the fixed effects of altitude, BCS category, sex and the interactions altitude × sex, BCS category × sex, altitude × BCS category, while herd was introduced as a random effect. Adjustment for multiple comparisons was done using the Bonferroni-method. In *study II*, a linear fixed effects model was used to detect differences in hepatic trace element concentrations

according to the visual presence of parasitic infection, investigating both absence versus presence of parasitic and differences between different types of infection. Pairwise differences were adjusted using Tukey's multiple comparisons technique. Significance was declared at a probability level of $p < 0.050$ while $0.050 \leq p < 0.100$ was interpreted as a trend.

2.3 Results

2.3.1. Study I

Average mineral plasma values of the zebu cattle grazing around the Gilgel Gibe catchment are presented in Table 2.1. Manganese concentrations in most plasma samples were below the detection limit, and are consequently, not presented here.

Table 2.1. Mean plasma mineral concentrations of zebu (*Bos indicus*) cattle (n = 90) grazing around the Gilgel Gibe catchment, Ethiopia.

							Diagnostic criteria				
Mineral	Mean			Range			Marginal[1]	Deprived[2]	Khalili *et al.*[3,4]		
Ca, mg/l	218	±	49	164	-	493	_	80	110	±	12
P, mg/l	102	±	17	57	-	143	_	46	61	±	13
Na, mg/l	3012	±	131	2725	-	3596	_	3219		_	
K, mg/l	173	±	28	106	-	257	_	98	153	±	21
Mg, mg/l	47	±	5	34	-	57	_	18	22	±	5
S, mg/l	1194	±	138	705	-	1506	_	_		_	
Fe, mg/l	8	±	7.4	4.2	-	67	1.3	1	2.2	±	0.4
Zn, mg/l	3	±	1.1	1.6	-	7	0.8	0.6	0.5	±	0.2
Cu, mg/l	0.38	±	0.27	0.05	-	1.15	0.7	0.57	0.57	±	0.22

± followed by standard deviation (SD)

[1]diagnostic criteria for marginal deficiency in *Bos taurus* cattle according to Kincaid (2000).

[2]upper marginal band for diagnostic criteria indicating a probable deprivation risk in *Bos taurus* cattle according to Suttle (2010).

[3]Khalili *et al.* (1993a): plasma macrominerals in *Bos indicus* cattle grazing in a comparable environment.

[4]Khalili *et al.* (1993b): plasma microminerals in *Bos indicus* cattle grazing in a comparable environment.

Mean plasma mineral concentrations of cattle grazing at the different altitude regions are shown in Figure 2.2. Plasma Ca and Cu concentrations were significantly lower in animals at lowest altitude compared to highest altitude ($p < 0.050$). For P and S, there was a trend towards lower concentrations in animals originating from the lowest and intermediate altitude, respectively, compared to animals from a different altitude ($p = 0.08$, $p = 0.09$, respectively).

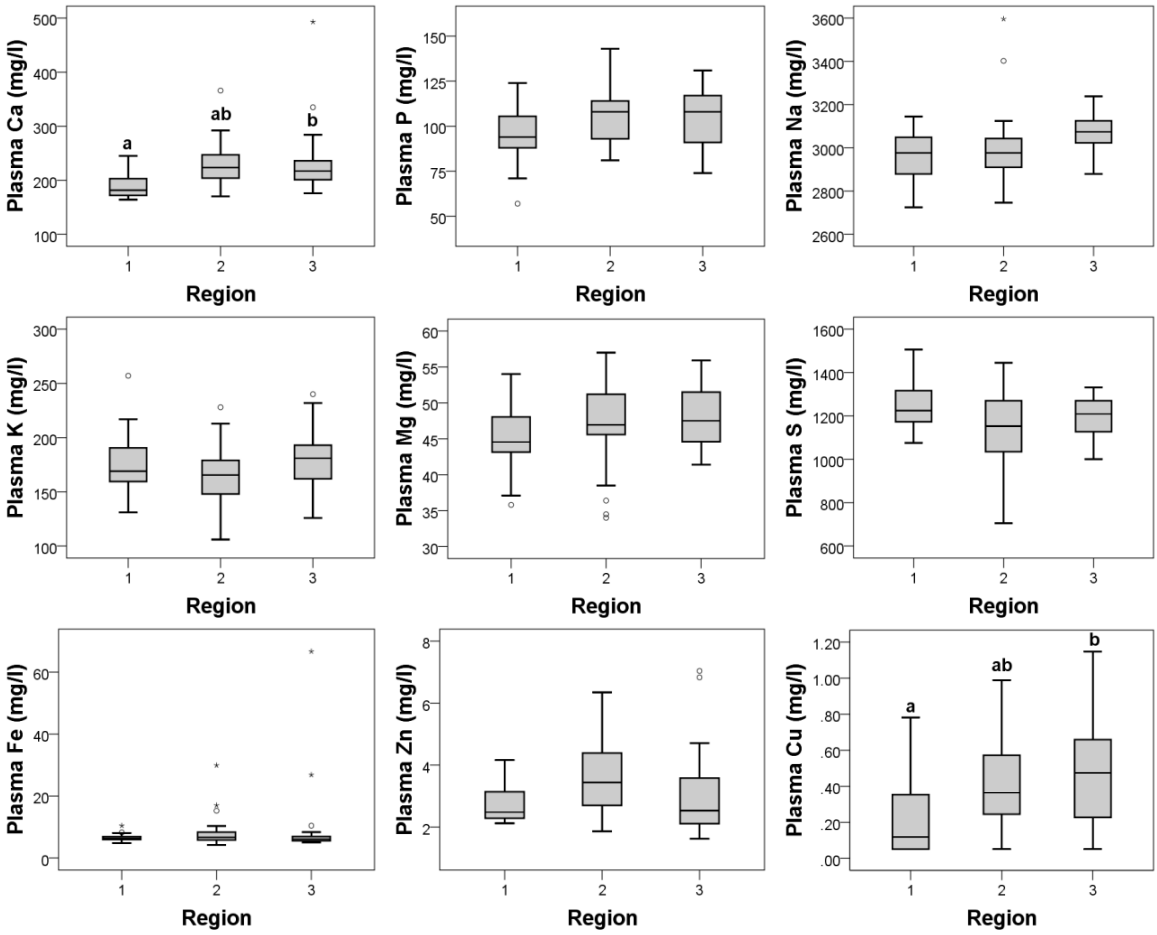

Figure 2.2. Box plots of plasma mineral concentrations in zebu (*B. indicus*) cattle (n = 90) grazing at different altitude subregions (1 = at < 1700 m asl, 2 = at 1700-1900 m asl, 3 = > 1900 m asl) around the Gilgel Gibe catchment, Ethiopia. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values. [a,b]Plasma Ca and Cu concentrations in cattle grazing at different altitude differed ($p < 0.050$).

Furthermore, animals assigned different body condition scores (Figure 2.3) differed in plasma Cu concentrations ($p < 0.050$), while a trend was present for K concentrations ($p = 0.05$), i.e. plasma Cu concentrations were higher in fat than in lean and medium

animals, whereas plasma K concentrations tended to be lower in fat than in lean animals.

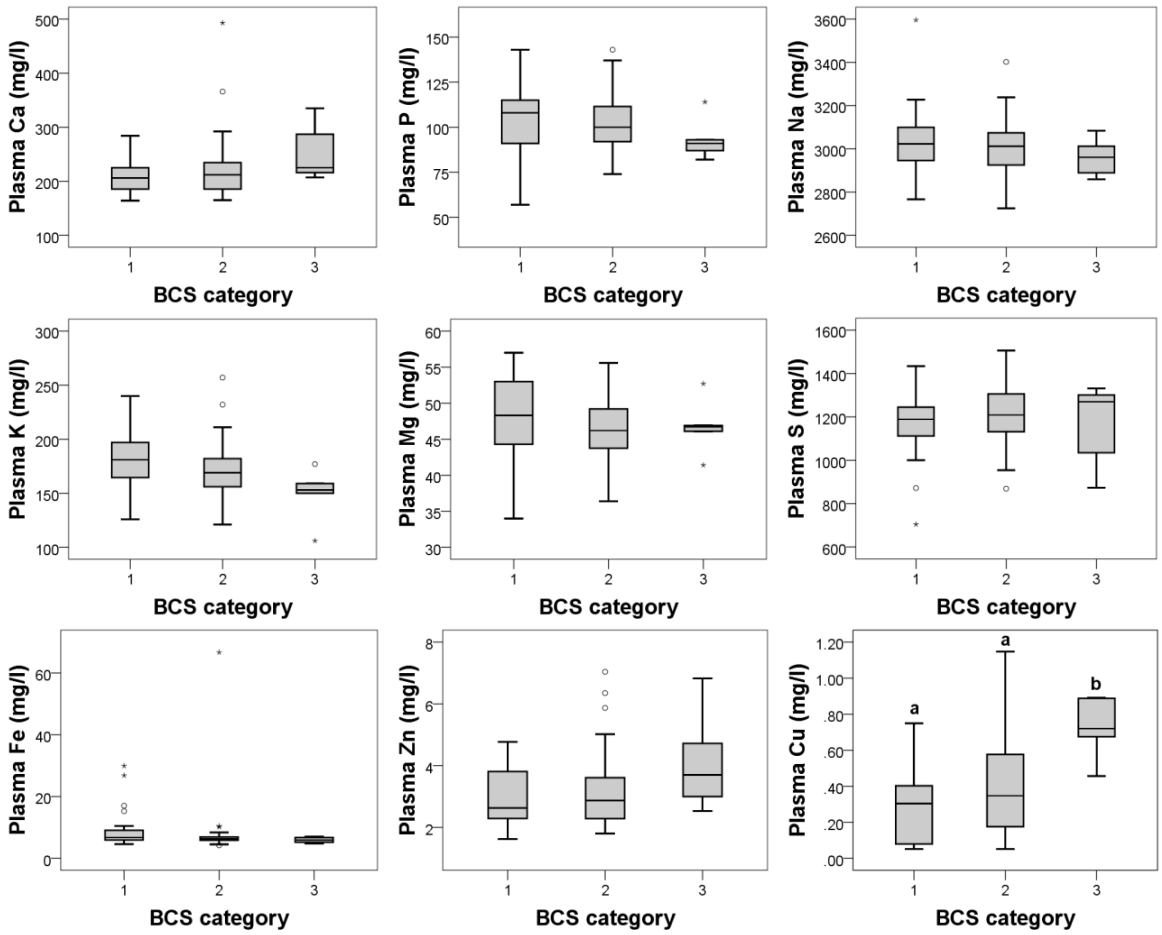

Figure 2.3. Box plots of plasma mineral concentrations in lean (1), medium (2) and fat (3) zebu (*B. indicus*) cattle (n = 90) grazing around the Gilgel Gibe catchment, Ethiopia (1= BCS 1, 2, 3; 2= BCS 4, 5, 6; 3= BCS 7, 8, 9; using the 1-9 point scale for zebu cattle, formulated by Nicholson & Butterworth (1986)). The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values. [a,b]Plasma Cu concentrations differed between animals of different BCS categories ($p < 0.050$).

When comparing sexes (Figure 2.4), a trend towards lower plasma Mg concentrations in bulls was present ($p = 0.09$), while none of the other plasma mineral concentrations differed between sexes. Plasma concentrations of Na were affected by a BCS category × sex interaction ($p < 0.010$), while an altitude × sex interaction tended to affect plasma Fe concentrations ($p = 0.07$). Subregional sex and BCS distribution was uniform ($p > 0.050$). The mean BCS over all subregions was 4.2 ± 1.5 (SD) according to the zebu scale of 1

(extremely lean) to 9 (extremely fat). Pooled herd faecal mineral concentrations are presented in Table 2.2.

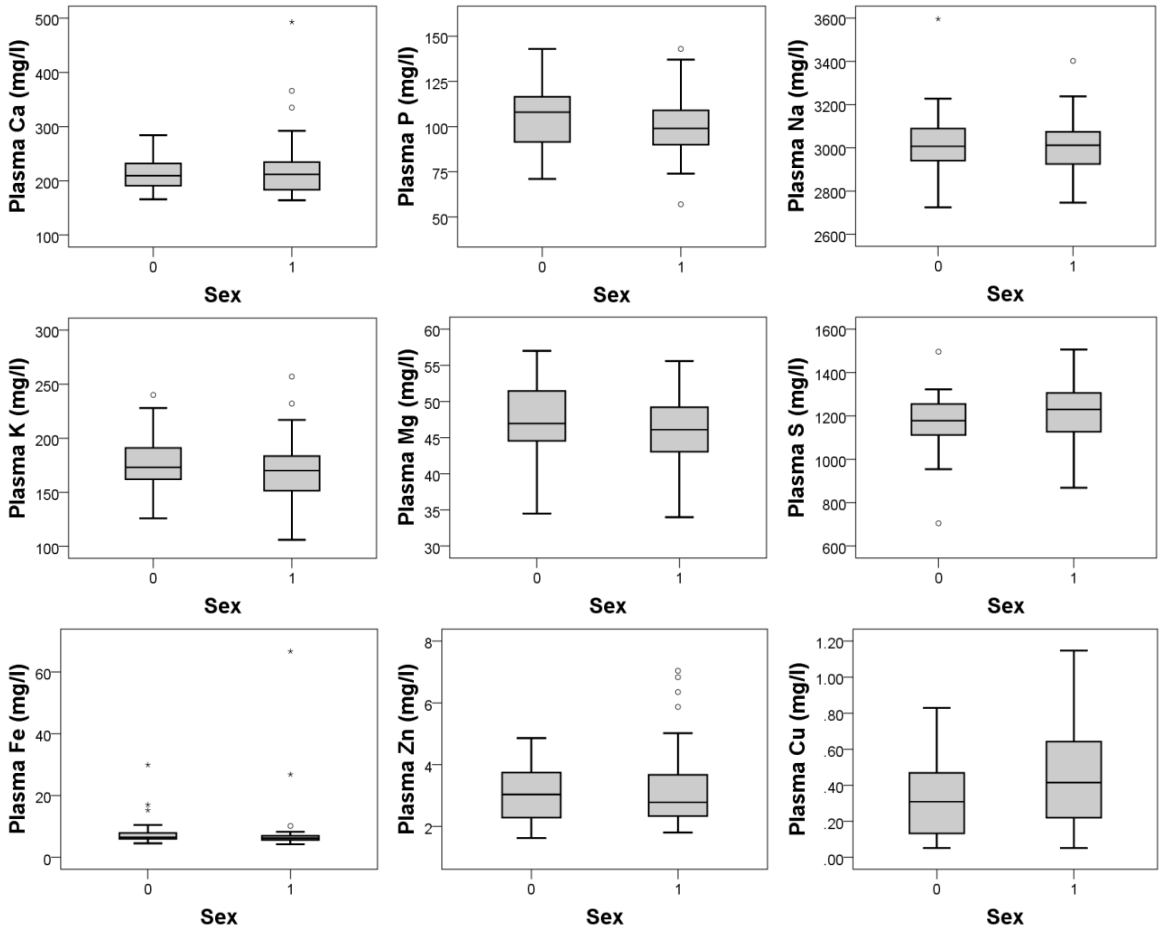

Figure 2.4. Box plots of plasma mineral concentrations in female (0) and male (1) zebu (*B. indicus*) cattle (n = 90) grazing around the Gilgel Gibe catchment, Ethiopia. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values.

Table 2.2. Mean faecal mineral concentrations (dry weight, DW) in herds of free-grazing zebu (*Bos indicus*) cattle (n = 18) around the Gilgel Gibe catchment, Ethiopia.

Mineral	Mean			Range			Khalili *et al.*[1,2]		
Ca	13	±	3	9.4	-	19	15.5	±	7
P	4.9	±	1	3.3	-	7	7	±	2.5
Na	0.6	±	0.2	0.3	-	1	1	±	1.5
K	12.5	±	1.3	9.7	-	13.5	18	±	12
Mg	3.1	±	0.7	2	-	4.3	5.5	±	3
S (g/kg DW)	0.87	±	0.2	0.61	-	1.19		_	
Fe	7054	±	2329	4089	-	12823	12374	±	8947
Zn	74	±	14	45	-	95	84	±	34
Cu	16	±	4	16	-	25	24	±	9
Mn (mg/kg DW)	887	±	280	492	-	1321	823	±	486

± followed by standard deviation (SD)
[1]Khalili *et al.* (1993a): faecal macrominerals in *Bos indicus* cattle grazing in a comparable environment.
[2]Khalili *et al.* (1993b): faecal microminerals in *Bos indicus* cattle grazing in a comparable environment.

2.3.2. Study II

Hepatic mineral concentrations of the zebu cattle sampled at the local abattoir are shown in Table 2.3.

Table 2.3. Hepatic trace element concentrations (dry weight, DW) of zebu (*Bos indicus*) bulls (n = 53) grazing around the Gilgel Gibe catchment, Ethiopia.

							Diagnostic criteria				
Mineral	Mean			Range			Marginal[1]	Deprived	Khalili *et al.*[3]		
Fe, mg/kg DW	388	±	234	172	-	1250	_	150	1012	±	1887
Zn, mg/kg DW	170	±	48	96	-	309	40	_	144	±	62
Cu, mg/kg DW	68	±	107	5	-	591	125	19	15	±	9
Mo, mg/kg DW	3.57	±	0.61	1.22	-	5.27	_	_		_	
Se, mg/kg DW	0.94	±	0.22	0.64	-	1.69	1.25	0.07		_	

± followed by standard deviation (SD)
[1]diagnostic criteria for marginal deficiency in *Bos taurus* cattle according to Kincaid (2000).
[2]upper marginal band for diagnostic criteria indicating a probable deprivation risk in *Bos taurus* cattle according to Suttle (2010).
[3]Khalili *et al.* (1993b): hepatic microminerals in *Bos indicus* cattle grazing in a comparable environment.

Concerning parasitic infections, *Ascaris* spp. infestation was identified in the liver of one animal, *Echinococcus granulosis* in 6 cases and *Fasciola* spp. in 38 cases while parasitic infections were not macroscopically identifiable in 8 livers. There were no differences in hepatic mineral concentrations between animals with and without parasitic infection as detected upon visual inspection, nor among animals infected with different parasite species (all $p > 0.100$) (Figure 2.5).

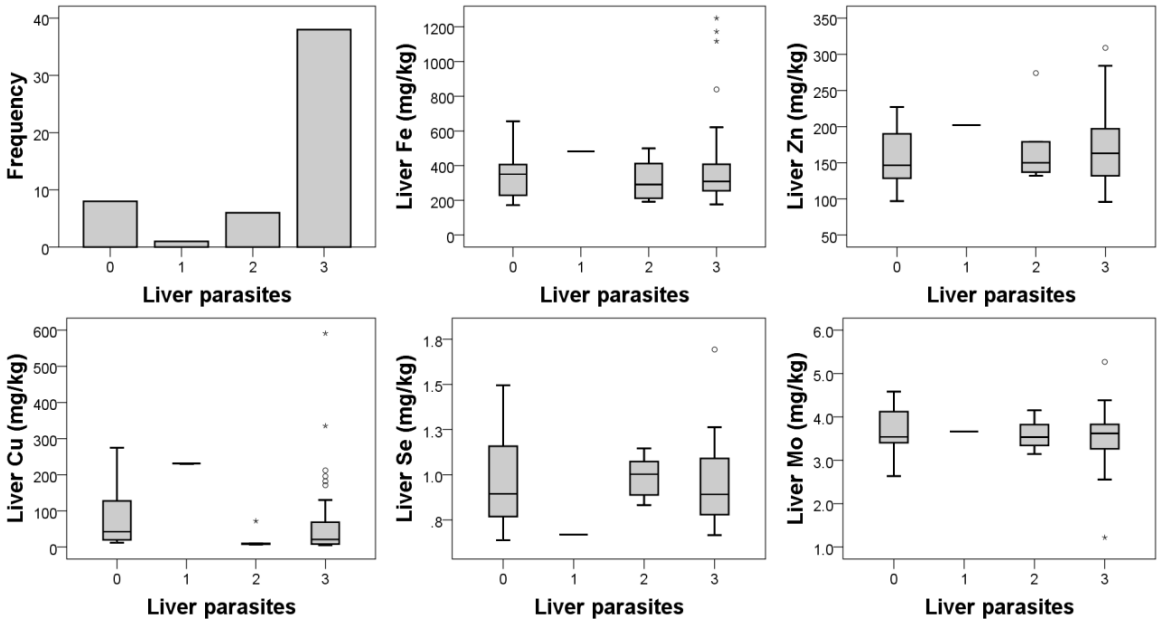

Figure 2.5. Frequency graph of presence of liver parasites as determined by visual inspection and box plots of hepatic mineral concentrations (dry weight) according to liver parasite (0= no visual detection of liver parasites, 1= *Ascaris* spp., 2= *Echinococcus granulosis*, 3= *Fasciola* spp.) in zebu (*Bos indicus*) cattle (n = 53) grazing around the Gilgel Gibe catchment, Ethiopia. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values.

2.4 Discussion

Plasma Cu concentrations in *study I* and hepatic Cu concentrations in *study II* suggest a shortage of Cu in grazing zebu cattle around the Gilgel Gibe region. Among the animals sampled in *study I*, 77% were at least marginally Cu deficient (< 0.70 mg/l, Kincaid, 2000), while 68% were probably Cu deprived (< 0.57 mg/l, Suttle, 2010), upon comparison of plasma Cu concentrations with diagnostic criteria for *Bos taurus* cattle found in

literature. Of the animals sampled at the local abattoir in *study II*, 81% were at least marginally Cu deficient (< 125 mg/kg dry weight (DW), Kincaid, 2000), while 47% were probably Cu deprived (< 19 mg/kg DW, Suttle, 2010), based on their hepatic Cu concentrations. In general, diagnostic criteria for mineral deficiencies in *B. indicus* cattle are absent and it is uncertain whether those stated for *B. taurus* cattle are applicable to zebu cattle, seeing that differences in mineral requirements exist even among *B. taurus* cattle breeds (Pogge *et al.*, 2012). Low plasma and hepatic Cu concentrations in cattle are in accordance with earlier research done in East Africa, e.g. by Gizachew *et al.* (2002), although it is not clear whether this was accompanied with clinical disorders or not. In *study I*, plasma Cu concentrations were lower in animals originating from low altitude and in cattle with a lower BCS. Although low altitude might relate to the soil type in this study area, the possible influence, if any, on the mineral status of the cattle grazing in the area should be further investigated. The lower BCS might have been a consequence rather than a cause of the lower Cu status, frequently associated with severe diarrhoea, growth retardation and anaemia (Suttle, 2010), but other factors such as differences in physiological status might also have affected both BCS and Cu status. In *study II*, a low hepatic Cu status was not associated with the presence of parasites in the liver, which is in contrast to the data from Vengušt *et al.* (2003) in fallow deer, whether applicable or not to cattle, who found lower Cu concentrations in livers infected with *Fasciola* spp..

Molybdenum concentrations in sampled cattle livers in *study II* were outside normal ranges for *B. taurus* cattle (1.0-4.0 mg/kg DW) as stated by Herdt & Hoff (2011) in 17% of cases. Molybdenum is known to induce secondary Cu deficiency through the formation of the insoluble thiomolybdate complexes with sulphur and in excess, thiomolybdates, can bind Cu at post absorption sites, causing thiomolybdate toxicity (Kincaid, 2000). Hepatic Mo concentrations, that are responsive to dietary changes (Gardner *et al.*, 2003), are being analysed in ruminants in order to investigate the possibility of excessive Mo intake (Steinke *et al.*, 2006). In the presence of high ruminal sulfide concentrations, relatively high dietary Mo concentrations can cause secondary Cu deficiency and thiomolybdate toxicity (Spears, 2003) and therefore, the high hepatic Mo concentrations could point to the potential presence of these conditions in at least some of the cattle in the area. However, the Cu, Mo and S interaction is complex and thiomolybdate synthesis

also occurs at lower dietary Mo concentrations (Spears, 2003). Consequently, more information on dietary S concentrations would be needed in order to fully predict the occurrence of these phenomena in grazing zebu cattle around the Gilgel Gibe region.

Iron overload has the potential to aggravate a situation of Cu shortage, given that the ingestion of high Fe concentrations (e.g. when prolonged soil ingestion is present) induces the formation of insoluble Fe-Cu-S complexes, leaving even less Cu available to engage in the thiomolybdate-Cu interactions mentioned above (Gould & Kendall, 2011). This effect lowers Cu absorption and hence, results in lower bovine plasma and hepatic Cu concentrations (Humphries *et al.*, 1983). Hepatic Fe concentrations above 1000 mg/kg DW are considered an indication of excess Fe intake (Suttle, 2010). In this study, 3 out of the 53 bulls had hepatic Fe concentrations above this threshold value. We can conclude that Fe overload might be present and interfering with Cu but seems to be a minor determining factor in the widespread Cu deficiency of cattle in the area.

Overall, plasma mineral concentrations of *study I* were higher than those recorded in the comparable environment of the Selale Highlands in Ethiopia by Khalili *et al.* (1993a,b), with the sole exception of lower plasma Cu concentrations. Generally, faecal mineral concentrations were lower than in Khalili *et al.* (1993a,b), while mean Fe levels in liver samples in *study II* were lower and hepatic Cu concentrations were higher compared to those values of Khalili *et al.* (1993b). This might indicate that the Cu deficiency in animals studied by Khalili *et al.* (1993b) was probably caused by an excess of Fe, whereas in our study, as mentioned above, the Cu deficiency was potentially caused by a primary Cu shortage possibly exacerbated by a Mo interaction.

Furthermore, none of the other mineral concentrations were found to be marginal or deficient in the plasma samples, except for Na, Mn and Se. Plasma Na concentrations of cattle in *study I* were deficient in 80% of the cases according to the marginal bands for deprivation for *B. taurus* cattle (< 3219 mg/l) stated by Suttle (2010). Plasma Na concentrations should be interpreted carefully, as lower values might be due to non-nutritional causes, such as profuse sweating and severe diarrhea while higher values might be due to dehydration (Suttle, 2010). Furthermore, the sodium heparin tubes used in this study may have falsely elevated sodium concentrations in our samples. However,

mean herd faecal Na values were mostly below 1000 mg/kg DW and the Na:K ratio of these samples were below 0.1, which is suggested by Little (1987) to be indicative of deprivation. The values were also lower than those reported by Khalili *et al.* (1993a) of *B. indicus* cattle grazing in a comparable environment. Tropical forages are indeed known to be insufficient in Na (McDowell and Arthington, 2005).

Claims concerning the presence of animals (marginally) deficient in Mn are difficult to substantiate given the large number of animals in *study I* with plasma Mn concentrations below the detection limit. Manganese concentrations in plasma are extremely variable and are not a reliable indicator of Mn status (Legleiter *et al.* 2005). However, Mn deficiency is rarely reported in adult ruminants and is not to be expected in this environment (McDowell & Arthington, 2005).

Of the livers sampled in *study II*, Se concentrations were marginally deficient in 92% of the cases according to the diagnostic criteria of Kincaid (2000). However, Se concentrations in our study were similar to those found by Frøslie *et al.* (1983) in Kenya, in animals with no clinical symptoms of deficiency. Also, the hepatic Se threshold value for marginal Se deficiency provided by Kincaid (2000) (< 1.25 mg/kg DM) lies well above the critical hepatic Se concentration mentioned by McDowell & Arthington (2005) (> 0.25 mg/kg DM) and the marginal ranges for deprivation as stated by Suttle (2010) (> 0.07 mg/kg DM) while well within normal ranges provided by Herdt & Hoff (2011) (0.70-2.5 mg/kg DM), indicating yet again the wide range of diagnostic criteria available for the evaluation of mineral status in cattle by means of tissue and blood analysis (Hall, 2005; Suttle, 2010; Herdt & Hoff, 2011).

The status of the remaining macro- and microminerals (Ca, P, K, Mg, Zn) is considered non-deficient, upon comparison with criteria for classification of *B. taurus* plasma and hepatic mineral concentrations found in literature (Kincaid, 2000; Suttle, 2010). However, plasma concentrations of Ca, Fe and Zn as well as Mn, for some animals, in *Study I* were substantially higher than normally observed in *B. taurus* cattle as well as *B. indicus* cattle in comparable environments (Khalili *et al.*, 1993b; Suttle, 2010). Zinc levels in this range would imply the presence of a strong zinc-copper antagonism through upregulation of metallothionein, thereby minimizing intestinal Cu absorption (Suttle,

2010). Nevertheless, the normal faecal values refute the possibility of an abnormally high Zn intake. Falsely elevated plasma concentrations of some elements are present in case of dehydration (Suttle, 2010), but the low plasma Na concentrations in our study contradict the presence of this condition. Another explanation could be haemolysis in some samples as concentrations of these minerals are particularly sensitive to this (Herdt & Hoff, 2011), although we did not observe a high number of such samples in our study. The exact cause of the elevated plasma concentrations of these elements remains therefore unclear.

Conclusion

Copper deficiency was identified in cattle grazing around the Gilgel Gibe catchment, Ethiopia, based upon plasma and hepatic mineral analyses in two studies. High hepatic Mo concentrations were observed whilst tissue concentrations of Se and Na were too low. In *study I*, plasma Cu concentrations were lower in cattle with a low to medium BCS as well as animals grazing at low altitude. Differences in plasma mineral concentrations between altitude groups were also seen for Ca, while trends towards differences existed for P and S. Plasma K concentrations tended to differ between cattle in different BCS categories whereas Mg concentrations tended to differ between sexes. No differences were present in hepatic mineral concentrations according to the presence of parasites in the liver in *study II*.

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3

Sulphur levels in saliva as an estimation of sulphur status in cattle: a validation study

Adapted from:

V. Dermauw, E. Froidmont, J. Dijkstra, J. De Boever, W. Vyverman, A.-E. Debeer, G. P. J. Janssens (2012). Sulphur levels in saliva as an estimation of sulphur status in cattle: a validation study. Archives of Animal Nutrition **66**, 507-513.

Effective assessment of sulphur (S) status in cattle is important for optimal health yet remains difficult. Rumen fluid S concentrations are preferred, but are difficult to sample under practical conditions. This study aimed to evaluate salivary S concentration as estimator of S status in cattle. Saliva and rumen fluid samples were collected from dairy cows (n = 16) as well as samples of different feedstuffs offered to the animals. The N and S concentrations were determined using the Dumas technique. The average dietary N and S content were calculated as well as N:S ratio of saliva, rumen fluid and diet. Salivary S concentrations were not found to be predictive for rumen fluid or dietary S concentrations (p > 0.050). The log transformed salivary N:S ratio (x) could predict the rumen fluid N:S ratio (y) with a linear equation of y = 9.83 (± 4.59) x + 0.39 (± 4.56) (r = 0.497, p = 0.050), but left too much residual variation to serve as reliable predictor. Further research should investigate this relationship in the search for a S status estimator.

3.1 Introduction

Copper and selenium deficiency are two of the most common trace element deficiencies in grazing cattle (McDowell & Arthington, 2005; **Chapter 2**). An important antagonist of both elements is sulphur (S). Consequently, high levels of this mineral in ruminant diets are to be avoided, since they can cause a secondary copper and selenium deficiency (Spears, 2003). Moreover, such high intake of sulphur can increase the risk of polioencephalomalacia (Gould, 1998).

However, this macro-mineral is also essential for ruminant health. Rumen bacteria convert sulphate to sulphide which can be incorporated in the S containing amino acids, methionine and cystein. Through this pathway, S is important for the production of microbial protein (Lewis, 1954). Therefore, S deficiency can lead to hypoalbuminaemia and decreased total serum protein (Ortolani, 2001). Furthermore, S is involved in synthesis of vitamins (thiamine and biotin), glutathione, in rigidity of proteins through disulphide bridges (in hair and hoofs) and in many coenzymes (Komarnisky *et al.,* 2003).

Sulphur deficiency in cattle might occur more often than expected. In tropical regions, soil contains low levels of S, due to heavy rainfall and high solubility of S salts (Leng, 1990). In industrialised areas, the incidence of S deficiency is increasing, caused by a reduced S content of soil fertilisers and as a surprising side-effect of global reduction of acid rain (Dick *et al.,* 2008).

Overall, the importance of an adequate but moderate S status for ruminants is clear. However, the assessment of S status remains problematic. Several estimators were investigated in the past, such as sulphate levels in plasma (Weir & Rendig, 1954), sulphide concentration in rumen fluid (Bray & Hemsley, 1969; Qi *et al.,* 1993) and total S in plasma (Jacobson *et al.,* 1967; McAdam & O'Del, 1982; Bawala *et al.,* 2009; **Chapter 2**), none of them rendering an accurate estimator of S status in cattle. Preferably, rumen fluid is used, considering the S available for microbial protein synthesis in the rumen is determining the optimal use of S (Suttle, 2010). In this context, a parameter linked with the rumen S metabolism but easier to sample, would considerably promote field work

on S status. Similar to urea in nitrogen (N) metabolism, S undergoes recycling mechanisms. Kennedy & Siebert (1972) stated that part of the S is recycled from rumen via blood to saliva which could add again to the rumen S metabolism. According to Suttle (2010), S levels in saliva decrease as dietary intake and plasma sulphate levels decline. Given its practicality, the S concentration in saliva is worthwhile investigating as an estimator of S status. The aim of this study is to analyse S concentrations in saliva and link them with S concentrations in rumen fluid and feed, thus allowing the validation of salivary S concentration as estimator of S status in cattle.

3.2 Materials and methods

3.2.1. Animals and diets

Sixteen adult Holstein Friesian cows fitted with permanent rumen cannulae were included in this study. All animals were selected from scientific projects and studies in other research centres which required them to be cannulated, so no animals were fitted with a cannula for the purpose of this study. Four animals were included from ILVO (Institute for Agricultural and Fisheries Research, Melle, Belgium), seven from CRA-W (Walloon Agricultural Research Centre, Gembloux, Belgium) and five from WUR (Wageningen University & Research Centre, Wageningen, The Netherlands). The experimental diets provided to the animals are characterized in Table 1. Prior to sampling, all animals were fed the described rations for at least two weeks in order to guarantee the steady state of their S metabolism.

3.2.2. Samples and analyses

A sample (approximately 50 ml) of the rumen fluid was collected through the cannula by the use of a vacuum pump and a tube. Saliva was sampled by placing a sponge (pre-rinsed with distilled water and wringed out) in the mouth of the animal and wringing it out in a recipient (Figure 3.1). In this way, a sample of mixed saliva (originating from the

different buccal saliva glands) was obtained without fistulation of glands, which implies a great practical advantage. All samples were taken between 6 and 8 h following the last concentrate meal, to avoid interference caused by the rapid postprandial rise of S compounds in saliva and rumen fluid (Bray and Hemsley 1969).

Figure 3.1. Sampling of cows (n = 16) to evaluate saliva as an indicator of sulphur status. **Left**: a cow with rumen canula. **Right**: saliva sampling

A representative sample was taken from all feedstuffs included in the diet and the offered quantities of these feedstuffs were recorded (Table 3.1). All samples were transported on dry ice and stored at -18 °C. Saliva and rumen fluid samples were freeze-dried and feed samples oven-dried at 65 °C.

Table 3.1. Composition of diets provided to the sampled cows.

	Diet	
Cow	Ingredients	S concentration (% DM)
1	Concentrate + soy + corn silage + grass silage	0.20
2, 3	Concentrate + corn silage + haylage + straw + supplements	0.15
4, 5, 6, 7	Concentrate + corn silage + hay	0.19
8	Concentrate + corn silage + haylage + straw + supplements	0.15
9	Concentrate + corn silage + grass silage + supplements	0.41
10	Concentrate + corn silage + grass silage + supplements	0.39
11	Concentrate + corn silage + grass silage + supplements	0.42
12, 13	Grass silage	0.43
14, 15, 16	Concentrate + soy + corn silage + haylage	0.15

DM = dry matter

Both N and S levels were determined using the Dumas method (Crosland *et al.,* 2001), S with a Flash 2000 Organic Elemental Analyser (Interscience, Louvain-la-Neuve, Belgium), N with an ANCA-SL (Automated Nitrogen Carbon Analyser-Solids and Liquids) interfaced with a SerCon 20-20 IRMS with SysCon electronics (SerCon, Cheshire, UK). The average dietary S and N content was calculated through the weighted average of feedstuff S and N concentrations, respectively. The division of the average dietary N content by the average dietary S content resulted in the dietary N:S ratio.

3.2.3. Statistical analyses

Normal distribution of data was tested by means of the Kolmogorov-Smirnov normality test with Lilliefors Significance Correction. Sulphur concentrations and N:S ratio in diet were non-normally distributed. Nitrogen concentrations in saliva, rumen fluid and diet, and the N:S ratio in rumen fluid were normally distributed. The S concentrations in saliva and rumen fluid and the N:S ratio in saliva were normally distributed after log transformation. Correlation coefficients were calculated using a Spearman correlation test in non-normally distributed and mixed data, and a Pearson correlation test in normally distributed data. Linear regression analysis was used to calculate the predictive value of parameters. All statistical procedures were executed in SPSS19 (SPSS Inc., Illinois, Chicago). Significance levels were set at $p < 0.050$, and a trend was considered at a probability of $0.050 \leq p < 0.100$.

3.3 Results

Nitrogen, sulphur concentrations and N:S ratio of mixed saliva, rumen fluid and total diet feed samples are summarised in Table 3.2. Nitrogen and S analyses were highly reproducible; the average standard deviation between replicates for N in saliva, rumen fluid and diet was 0.10, 0.08 and 0.14 mg/100 mg dry matter (DM) respectively, for S in all samples 0.02 mg/100 mg DM.

Table 3.2. Nitrogen and sulphur concentration and N:S ratio of mixed saliva, rumen fluid and total ration samples of dairy cattle (n = 16).

Variable	Sample	Mean	SD	Range
N (mg/100 mg DM)	Mixed saliva	3.87	1.12	2.36-5.93
	Rumen fluid	4.07	1.04	1.94-6.12
	Total diet	2.27	0.60	1.30-2.76
S (mg/100 mg DM)	Mixed saliva	0.56	0.44	0.10-1.73
	Rumen fluid	0.67	0.48	0.16-1.60
	Total diet	0.25	0.12	0.15-0.43
N:S ratio	Mixed saliva	11.59	9.68	2.58-38.19
	Rumen fluid	9.59	6.88	2.10-28.87
	Total diet	10.97	5.32	5.84-18.30

SD= standard deviation, DM= dry matter

A trend towards a positive correlation between the log transformed S concentrations in saliva and rumen fluid (r = 0.430, p = 0.096) was observed. The relationship between the log S concentrations in saliva and rumen fluid is illustrated in Figure 3.2. Furthermore, the log S concentrations in rumen fluid as well as in saliva did not correlate well with the dietary S concentrations (all p ≥ 0.100).

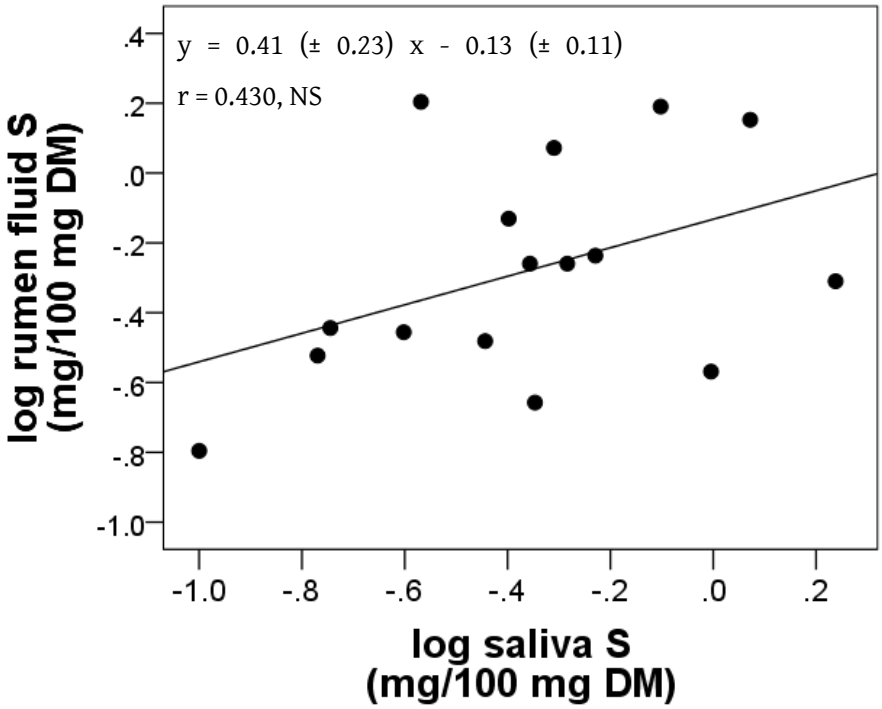

Figure 3.2. The relationship between the log salivary S concentration and the log rumen fluid S concentration in dairy cattle (n = 16). NS, non-significant

The log salivary N:S ratio (x) provided a near significant, but weak prediction of the N:S ratio in rumen fluid (y) ($r = 0.497$, $p = 0.050$), with a linear equation of $y = 9.83 (\pm 4.59) x + 0.39 (\pm 4.56)$ (Figure 3.3). Neither the log N:S ratio in saliva, nor the N:S ratio in rumen fluid was correlated with the N:S ratio of the diet (all $p \geq 0.100$).

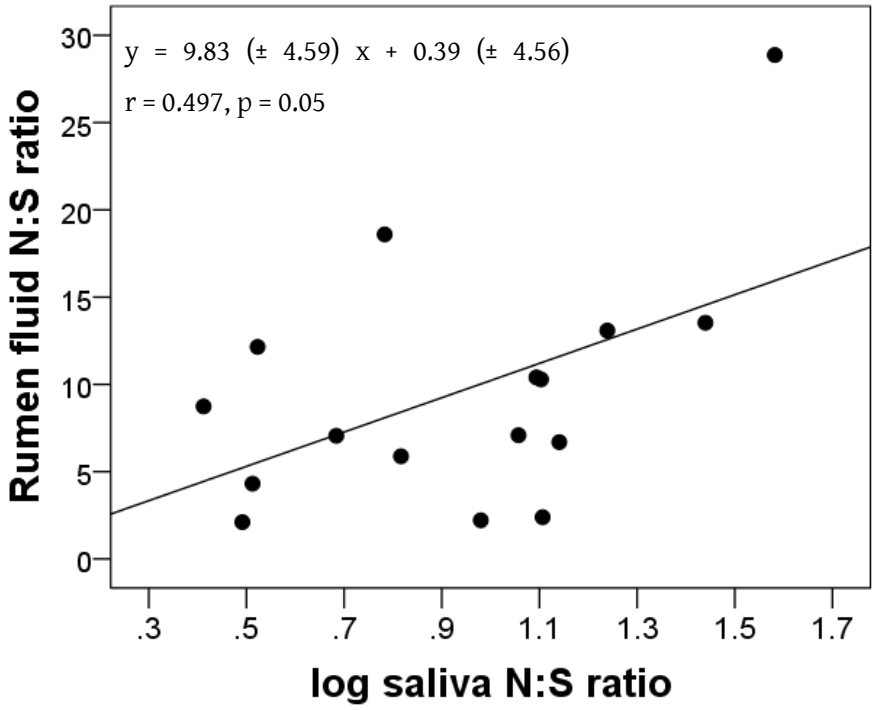

Figure 3.3. The relationship between the log salivary N:S ratio and N:S ratio in rumen fluid in dairy cattle (n = 16).

The rumen fluid N concentration was not well predicted by the salivary N concentration ($r = 0.406$, $p \geq 0.100$). Finally, both the salivary and rumen fluid N concentrations did not correlate well with the dietary N concentrations (all $p \geq 0.100$).

3.4 Discussion

Our data for N concentrations in saliva and rumen fluid are somewhat higher than data from earlier research, such as 0.9-3.6 mg/100 mg DM (McDougall, 1948) and 1-2 mg/100

mg DM (Davis & Stallcup, 1967) for N concentrations in saliva and rumen fluid, respectively. The S concentrations we measured are much higher than those reported in literature (e.g. 0.035-0.112 mg/100 mg DM in saliva (Doyle *et al.,* 1982), 0.29-0.41 mg/100 mg DM in rumen fluid (Evans & Davis, 1966). However, in the literature, several methods for S are considered valid and are used for determination of total sulphur, such as induction coupled emission spectrometry, colorimetry, gravimetry, ion chromatography, Dumas method (Crosland *et al.*, 2001) and turbidimetry (Van Ranst *et al.,* 1999). This renders a comparison of our data with earlier research difficult. Crosland *et al.* (2001) showed high variability between methods and between laboratories for S concentration and concluded that the reliability of N:S ratio was limited by the accuracy of the S rather than the accuracy of N analysis. Nevertheless, it was not our intention to evaluate absolute numbers in our study, rather it was our objective to investigate whether variations in salivary S concentrations corresponded with fluctuations in S concentrations in rumen fluid and diet.

The apparent weak relationship ($p = 0.096$) between S concentrations in saliva and in rumen fluid is partly in line with Bray & Hemsley (1969). They reported a significant increase in both salivary S and rumen fluid sulphide-S concentrations when dietary S concentration increased from 0.058 to 0.143 mg/100 mg DM, but upon a further increase to 0.318 mg/100 mg DM, salivary S concentration did not increase further whilst rumen fluid sulphide-S concentrations increased. In the present dataset, all of the dietary S concentrations are above 0.143 mg/100 mg DM and thus above the value in Bray & Hemsley (1969) at which salivary and rumen fluid S concentrations increased. Although the salivary S recycling theory is well established (Bray & Hemsley, 1969; Kennedy *et al.,* 1975; Kandylis, 1983; Durand & Komisarczuk, 1988) and cattle are considered to recycle S more efficiently than sheep (Bird, 1974), there is a paucity in research on associations between salivary S and rumen fluid S, and between salivary and dietary S concentrations. In contrast, in various studies a significant positive relationship between rumen fluid and dietary S concentrations was established (Evans and Davis, 1966; Kennedy & Siebert, 1972; Bawala *et al.* 2009), but in the present study, the relationship between dietary and rumen fluid S concentrations was not significant. While our research does not indicate a significant correlation between rumen fluid and

saliva concentrations of S mutually nor between these parameters and dietary S concentration, this does not exclude the presence of a dietary-rumen fluid-saliva cycle in the animals studied. The mean dietary S concentration was 0.25 mg/100 mg DM, which is above the requirements for dairy cattle of 0.20 mg/100 mg DM (NRC, 2001). Possibly, the dietary S concentrations were not low enough, and consequently, the recycling not intensive enough to allow saliva to be a reliable indicator for S status in the cows in this study. Moreover, validating an estimator for a wide range of practical applications in the field requires an evaluation of this estimator over different farming conditions. Obviously, this also might explain the lack of strong association between dietary, salivary and rumen fluid S concentrations in contrast to studies under controlled conditions, with a single set-up and ration type.

Nevertheless, the relationship between the N:S ratio in saliva and the N:S ratio in rumen fluid warrants further research, in view of the fact that a proper ratio of dietary N and S is required for efficient microbial protein synthesis in the rumen (Kandylis, 1983). For dairy cattle, a N:S ratio between 10:1 and 12:1 is recommended (NRC, 2001), under the condition that both N and S are available for rumen microbes (Kandylis, 1983). However, it is clear that more factors need to be identified that affect salivary S concentrations before the latter can be used as a sufficiently sensitive and accurate estimator of S status in cattle.

Acknowledgements

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4

Effects of trace element supplementation on apparent nutrient digestibility and utilisation in grass-fed zebu (*Bos indicus*) cattle

Adapted from:

V. Dermauw, K. Yisehak, E. S. Dierenfeld, G. Du Laing, J. Buyse, B. Wuyts and G. P. J. Janssens (2013). Effects of trace element supplementation on apparent nutrient digestibility and utilisation in grass-fed zebu (*Bos indicus*) cattle. Livestock Science **155**, 255-261.

Trace element deficiencies in cattle are omnipresent, both in developing and industrialized regions. Little information is available on the effect of dietary trace elements on nutrient digestibility and utilisation, in spite of many deficiency-related symptoms suggesting a relevant role, such as loss of appetite in Zn deficiency and severe diarrhoea in Cu deficiency. The present study aimed to identify the early effects of dietary trace elements on nutrient utilisation in grass-fed zebu (Bos indicus) cattle. Adult bulls (n = 8) were randomly assigned to a treatment: control or trace element supplementation (Zn, Mn, Cu, Se, I and Co) during 28 days. Grass mineral analysis suggested deficient Cu (5.53-9.60 mg/kg) and Se (0.02-0.09 mg/kg) concentrations in combination with high S (2577-3855 mg/kg) and Mo (1.52-3.12 mg/kg) and very high Fe (619-1214 mg/kg) concentrations. Supplementation increased plasma Cu (0.82 vs. 0.61 mg/l), Zn (1.40 vs. 0.89 mg/l), Mn (0.30 vs. 0.05 mg/l) and Se (0.07 vs. 0.06 mg/l) concentrations (all $p < 0.050$). Faecal Cu, Zn, Mn and Se were also increased ($p < 0.050$), as was faecal Co ($p = 0.050$) concentration in supplemented bulls. On the contrary, trace element supplementation did not affect plasma ceruloplasmin and superoxide dismutase activities ($p > 0.050$). Also, no effects on apparent nutrient (dry matter, ash, protein, fat, fibre) digestibility, apparent trace element absorption (except for Se and I) or plasma acyl carnitines (indicators of available energy substrates) were observed in this study (all $p > 0.050$). Overall, despite clear improvement in trace element status - notwithstanding high concentrations of Cu antagonists in the grass diet – supplementation did not affect nutrient digestibility or utilisation in grass-fed zebu cattle..

4.1 Introduction

Studies in different regions of the world demonstrated that the concentrations of trace elements in pasture and rangelands can vary considerably with season, and often drop to levels of concern for deficiencies in animal nutrition (Blanco-Penedo *et al.*, 2009; Khalili *et al.*, 1993; Khan *et al.*, 2008). Optimal trace element supply is well known to be essential in ruminants for health and production. For example, Zn is important for reproduction and skin health, Fe for oxygen transport in the body and Cu for optimal immunity (Suttle, 2010). Several studies linked the presence of trace element deficiencies with the increased incidence of a whole range of diseases, such as chronic metritis, subclinical mastitis and lameness in cattle herds; these deficiencies were more frequent when cows were not supplemented with minerals (Guyot *et al.*, 2009; Mulligan *et al.*, 2006).

Certain functions of trace elements and deficiency-related symptoms are specifically associated with the digestive system. Copper deficiency is frequently accompanied by severe diarrhoea (McDowell & Arthington, 2005), while down-regulation of the Cu-dependent lysyl oxidase (EC 1.4.3.13) leads to impaired cross-linking of collagen accompanied with damaged gastro-intestinal connective tissue and ulceration (Frank, 1998), which is well known in humans to be associated with malabsorption syndrome (Jensen, 2000). Zinc on its behalf, is known to play a key role in DNA synthesis (Miller *et al.*, 1985) and therefore, deprivation of this element is most marked in rapidly dividing cells, such as intestinal cells. Consequently, Zn supplementation was able to cure intestinal damage in rats (Tran *et al.*, 2003). Furthermore, one of the first symptoms of Zn deficiency is loss of appetite (Suttle, 2010). Manganese is linked with lipid and carbohydrate metabolism through the activity of pyruvate carboxylase (EC 6.4.1.1), responsible for the conversion of pyruvate to oxaloacetate, the latter an important intermediate in the citric acid cycle, crucial in the cellular energy metabolism (NRC, 2000).

The previous paragraph suggests an important role of trace elements in digestive system function and nutrient utilisation. However, to the best of our knowledge, little

research has been conducted on the degree to which trace element status affects cattle nutrient digestibility and utilisation. One published study (Grace *et al.*, 2002), performed with grazing horses, reported no effect of trace element supplementation on the digestibility of proximate components (Grace *et al.*, 2002). Given the widespread occurrence of trace element deficiencies in grazing cattle around the world, the described biochemical and physiological association of trace elements with digestive system function and the lack of relevant studies in cattle, our objective was to evaluate the early (< one month) effects of trace element supplementation on nutrient digestibility and utilisation in grass-fed zebu (*Bos indicus*) cattle, naturally varying in trace element supply.

4.2 Materials and methods

4.2.1. Animals and housing

This study was reviewed and approved by the Ethical Commission of the Faculty of Veterinary Medicine at Ghent University (EC: Case 2010_102). The trial was conducted at Jimma University College of Agriculture and Veterinary Medicine, Ethiopia. Eight adult Ethiopian highland zebu (*B. indicus*) bulls were obtained from a local livestock market, in a region with cattle displaying trace element deficiencies as established by previous work (**Chapter 2**).

All bulls were aged between 4 and 6 years (mean: 4.9 year ± 0.2) and weighed between 139 and 189 kg (mean: 163 kg ± 7). The animals had a body condition score between 3 and 7 (mean: 4.6 ± 0.46) on a scale of 1 to 9 designed for zebus (Nicholson and Butterworth, 1986). The bulls were housed in separate stables. After arrival, the animals were weighed and dewormed using a combination of ivermectine and clorsulon (Ivomec F®, 0.02 ml/kg body weight, Merial Animal Health, Brussels, Belgium) and left to adapt to the housing conditions and diet for one week.

Table 4.1. Proximate, fibre and mineral analysis of the grass diet

	Experimental week[1]						
Parameter	1	2	3	4	5	SE	p
(g/kg)							
DM	183	208	209	244	209	10	0.52
(g/kg DM)							
Ash	131[a,b]	136[a,b]	119[a,b]	103[b]	140[a]	4	0.03
EE	22[a,b]	25[a]	18[b]	16[b]	19[a,b]	1	0.01
CP	133	149	137	94	132	7	0.07
NDF	631	614	679	615	648	9	0.14
ADF	346	319	353	355	338	7	0.59
ADL	63	58	76	61	54	6	0.88
(mg/kg DM)							
S	2601	2899	2963	2577	3855	164	0.06
Mo	1.5	2.1	3.1	2.3	1.9	0.2	0.10
Fe	619	2082	1214	879	777	210	0.18
Mn	114[b]	175[b]	189[a,b]	383[a]	233[a,b]	29	0.01
Zn	44[a,b]	53[a,b]	67[a]	34[b]	51[a,b]	4	0.04
Cu	5.6[b]	8.6[a]	9.6[a]	5.5[b]	5.8[b]	0.5	0.001
I	0.7	0.3	0.3	2.0	3.1	0.5	0.34
Se	0.02	0.07	0.05	0.09	0.09	0.01	0.14
Co	0.4	1.1	0.8	0.6	0.5	0.1	0.14

SE= standard error, , DM = dry matter, EE = ether extract, CP = crude protein, NDF = neutral detergent fibre, ADF = acid detergent fibre, ADL = acid detergent lignin

[1]week 1: unsupplemented grass diet in both groups, week 2 to 5 : unsupplemented grass diet in control group, in the supplementation group, grass diet supplemented with a tablespoon of molasses and trace element mix, providing (per kg DM) : 30 mg of Zn, 20 mg of Mn and 10 mg of Cu, as chelated-to-glycine forms, 0.10 mg of Se, as premix (all previous: MAAC®, Novus International (St.-Charles, Missouri, USA)), 0.50 mg of I as KI and 0.10 mg of Co as Co(NO3).6H2O.

[a,b]Significantly different between weeks at $p < 0.050$

4.2.2. Diet and supplementation

Throughout the experimental period, all bulls received a local grass mixture diet in order to simulate their natural diet with varying amounts of trace elements. The quantity of grasses supplied to the bulls was based on 2% of the individual body weight (McDowell, 1996), which was weekly monitored (Figure 4.1), and an average dry matter (DM) content of 20% in the grass. The nutrient composition of grass is presented in Table 4.1. Baseline measurements without treatment were executed in week 1. In week

2, the animals (n = 8) were randomly allocated to a treatment: trace element supplementation or control. Supplementation consisted out of a trace element mix supplying per kg DM of grass: 30 mg of Zn, 20 mg of Mn, 10 mg of Cu, all as chelated to glycine MAAC®; 0.1 mg Se as MAAC® Se Premix (Novus International, Inc., St. Charles, Missouri, USA); 0.1 mg of Co as $Co(NO_3)_2.6H_2O$ (1025360100, Merck, Overijse, Belgium) and 0.5 mg of I as KI (207969-100G, Sigma-Aldrich, Bornem, Belgium), according to the recommendations for beef cattle established by NRC (2000). The mineral powder was mixed with a spoon of molasses and top dressed on the offered grass to ensure complete intake.

4.2.3. Samples and storage

The experiment lasted for five weeks (baseline measurements without treatment followed by four treatment weeks). At the end of every week, during three subsequent days, apparent nutrient digestibility and apparent trace element absorption (see below) were estimated through total faecal collection: faeces were collected using faecal collection bags and grass refusals were weighed (Figure 4.1).

Figure 4.1. Sampling and measurement procedures during a trial investigating the effect of trace element supplementation on nutrient digestibility in grass-fed zebu bulls (n = 8). **Left:** faecal collection bags, **Right**: weighing bulls.

During these three days, daily subsamples of offered grass and faeces per animal were taken and afterwards faeces were individually pooled per faecal collection period. Faecal and grass samples were oven-dried at 65° C for 72 hours and ground through a 2 mm screen. From each animal, a weekly jugular blood sample was obtained using 20 G

needles (MN-2038M) and two sodium heparin tubes (VT-100SH, both Venoject®, Terumo, Leuven, Belgium). Plasma was obtained through centrifugation at 1500 × *g* for 10 min. All samples were stored at -20°C until further analysis.

4.2.4. Mineral analyses

As a preparation step for mineral analysis, faecal and grass samples were ashed through microwave destruction with 10 ml HNO_3 in closed vessels followed by filtration. Afterwards, the faecal and grass samples were analysed for Zn, Cu, Fe and Mn concentrations through inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX radial, Varian, Palo Alto, USA) and for Co, Se, I and S by means of inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, Perkin Elmer, Sunnyvale, USA). Plasma samples were prepared for mineral analysis through microwave destruction with 10 ml HNO_3 in open vessels followed by filtration and analysed for Cu and Zn concentrations through ICP-OES and Mn and Se concentrations through ICP-MS.

Throughout mineral analyses, a quality control programme was in use. Sampled matrices were spiked with elements under study with two concentrations in the range of the measured concentrations and recoveries were measured. Average recovery was 94%, with a range between 82% (Cu in faeces) and 113% (Se in plasma), which should probably be attributed to matrix interferences. Detection limits in acid digest were: Mn 0.35 µg/l, Cu 0.25 µg/l, Mo 0.33 µg/l, Se 0.13 µg/l, Fe 21.4 µg/l, Zn 16.4 µg/l, Co 0.14 µg/l. Standards were regularly run between samples. Analytical results were blank-corrected. Prior to use, all glassware and microwave vessels were rinsed with diluted HNO_3. Ultrapure HNO_3 (analytical grade for trace elements) was used during all analytical procedures.

4.2.5. Proximate and fibre analysis and digestibility calculation

Faecal and grass samples were also analysed for dry matter (920.36), crude protein (984.13), crude ash (923.03) and crude fat (920.39) by means of proximate analysis

(AOAC, 2000). Additionally, acid detergent fibre (ADF) and acid detergent lignin (ADL) were analysed according to AOAC (2000; 973.18) and neutral detergent fibre (NDF) by a method of Van Soest *et al.* (1991). Apparent nutrient digestibility as well as apparent mineral absorption were calculated as following: $((W_{diet} * C_{diet})-(W_{faeces} * C_{faeces})) / (W_{diet} * C_{diet}) * 100$ where W is the collected weight, C is the nutrient concentration (both on dry matter basis), *diet* refers to the offered diet including supplement after subtracting refusals, *faeces* refers to the total faecal output.

4.2.6. Plasma enzyme and acyl carnitines analysis

Moreover, plasma was analysed for the mineral dependent enzymes ceruloplasmin (EC 1.16.3.1) with the p-phenylenediamine oxidase method (Sunderman and Nomoto, 1970) and total superoxide dismutase (SOD) (EC 15.1.1) by means of the inhibition of WST-1 to WST-1 formazan reduction reaction (Peskin & Winterbourn, 2000). The latter reaction measured total superoxide dismutase activity in plasma, according to earlier research consisting mainly of EC-SOD (SOD3) (EC 15.1.1), a Cu and Zn-containing tetramer, and to a lesser degree of CuZnSOD (SOD1) (EC 15.1.1) and MnSOD (SOD2) (EC 15.1.1) as a result of leakage out of cells (Marklund, 1984). Finally, acyl carnitines analysis was performed through quantitative electrospray tandem mass spectrometry (Rizzo *et al.*, 2003). The plasma acyl carnitine profile served as reflection of the mitochondrial acetyl-CoA pool and thus, the available energy substrates for the citric acid cycle (Brass and Hoppel, 1980; Bremer, 1983).

4.2.7. Statistical analysis

All statistical analyses were performed using SPSS v17.0 (SPSS Inc., Chicago, IL, USA). Missing data (1 value for faecal mineral concentrations and 1 value for plasma acyl carnitines, ceruloplasmin and SOD) were replaced with the mean of the non-missing values of all individuals at the specific time point (Gadbury *et al.*, 2003). All data except feed composition were fit to a repeated measures model with treatment, time and their interaction inserted as fixed effects and baseline measurements (week 1) as covariate.

Individual animals served as experimental unit. Feed composition data were analysed using one-way analysis of variance. Significance was declared at a probability level of $p < 0.050$; $p < 0.100$ was interpreted to indicate a trend.

4.3 Results

Dietary concentrations of ash, fat, Cu, Zn and Mn differed significantly over the experimental period (Table 4.1). Mineral analysis of the grasses indicated a suboptimal supply of Cu (< 10 mg/kg DM) and Se (< 0.10 mg/kg DM) throughout the whole trial, while I was too low in week 2 and 3 (< 0.50 mg/kg DM), upon comparison with recommendations for *Bos taurus* beef cattle established by NRC (2000). According to Suttle (2010), Fe is antagonistic towards Cu when the Fe:Cu ratio is exceeding 50 whereas S and Mo are significantly depressing Cu absorption when S is higher than 2000 mg/kg DM combined with a Cu:Mo ratio lower than 3. Diet analysis values of all three elements showed values above these thresholds: for Fe constantly, for S and Mo in week 4, respectively.

At the onset of the study, three out of eight bulls had plasma Cu concentrations below disorder risk values for *B. taurus* cattle according to Suttle (2010), whereas two for Mn, and one for Zn. Supplementation of trace elements resulted in increased plasma concentrations of Cu, Zn, Mn and Se (Table 4.2), even though mean baseline and estimated marginal means of plasma mineral concentrations were above these risk values in both groups. Cu, Zn and Mn plasma values were also affected by a time × supplement interaction (all $p = 0.001$) (data not shown in Table 4.2).

Faecal concentrations of Cu, Zn, Se and Mn were raised by trace element supplementation (all $p < 0.050$), whereas faecal Co concentrations tended to be higher in the supplemented group ($p = 0.050$), faecal I concentrations, as an exception within the supplemented minerals, were not affected by the supplementation ($p > 0.050$).

Table 4.2. Trace element supplementation effects on estimated marginal means of plasma and faecal mineral concentrations in grass fed zebu (*Bos indicus*) bulls.

		Treatment				
Parameter	Baseline[1]	Control[2]	Supplement[2]	SE	p	Reference values[3]
Plasma mineral, mg/l						
Mn	0.031	0.053	0.297	0.025	0.002	>0.020[a]
Zn	0.89	0.89	1.40	0.08	0.006	>0.60
Cu	0.61	0.61	0.82	0.03	0.004	>0.60
Se	0.070	0.059	0.070	0.002	0.017	>0.020
Faeces mineral, mg/kg DM						
Mn	441	520	575	14	0.027	_
Zn	127	138	196	7	0.001	_
Cu	16	19	35	2	0.001	_
I	1.0	1.3	1.7	0.1	0.156	_
Se	0.13	0.21	0.36	0.02	0.004	_
Co	2.0	1.4	1.6	0.1	0.050	_

SE = standard error, p = p-value at significance level $p < 0.050$.
[1]Baseline= mean baseline concentrations (n = 8) in week 1
[2]Estimated marginal means of the treatment group= means per treatment over all repeated measures of week 2 to 5 adjusted for the baseline measurements in week 1 (inserted in the model as a covariate).
[3]from Suttle (2010)
[a]reference value for serum, no reference value available for plasma.

Mineral related ceruloplasmin and SOD activity remained unaffected by supplementation (Table 4.3). Mean baseline and estimated marginal means of ceruloplasmin activities in both treatment groups were substantially higher than the threshold value for Cu deficiency of 15 U/l suggested for *B. taurus* cattle by Laven *et al.* (2007).

Table 4.3. Trace element supplementation effects on estimated marginal means of plasma mineral related enzyme activity in grass fed zebu (*Bos indicus*) bulls.

		Treatment			
Enzyme activity	Baseline[1]	Control[2]	Supplement[2]	SE	p
Ceruloplasmin (U/l)	131	100	121	13	0.381
Superoxide dismutase (U/ml)	6.1	5.8	5.6	0.7	0.875

SE = standard error, p = p-value at significance level $p < 0.050$.
[1]Baseline= mean baseline enzyme activity (n = 8) in week 1
[2]Estimated marginal means of the treatment group= means per treatment over all repeated measures of week 2 to 5 adjusted for the baseline measurements in week 1 (inserted in the model as a covariate).

Trace element supplementation affected apparent digestibility neither of the proximate components nor of the fibre fractions ($p > 0.050$) (Table 4.4). A time × supplementation interaction affected apparent DM, crude protein and ADL digestibility (all $p < 0.050$) while apparent ash ($p = 0.09$) and NDF ($p = 0.07$) digestibility tended to be affected by this interaction. This interaction seemed to originate from a major and consistent shift in effect size between the two groups in week 3 of supplementation (data not shown in Table 4.4). Apparent Se and I absorption were increased in the supplemented group (both $p < 0.050$). Apparent Mn and I absorption were affected by time × supplementation interaction (both $p < 0.050$) and the same interaction tended to affect apparent Cu and Zn absorption ($p = 0.07$; $p = 0.08$, respectively) (data not shown in Table 4.4).

Table 4.4. Trace element supplementation effects on estimated marginal means of apparent nutrient digestibility and apparent mineral absorption in grass fed zebu (*Bos indicus*) bulls.

			Treatment			
Parameter		Baseline[1]	Control[2]	Supplement[2]	SE	p
Apparent nutrient digestibility (%)	DM	80	76	74	2	0.513
	Ash	62	61	58	3	0.577
	EE	84	70	69	1	0.461
	CP	84	79	77	2	0.401
	NDF	79	72	72	2	0.955
	ADF	77	69	68	2	0.780
	ADL	50	34	22	4	0.135
Apparent mineral absorption (%)	Mn	20	46	41	3	0.412
	Zn	41	32	36	6	0.699
	Cu	40	35	49	6	0.320
	I	70	51	74	4	0.021
	Se	-13	12	62	9	0.026
	Co	5	54	50	4	0.631

DM = dry matter, EE = ether extract, CP = crude protein, NDF = neutral detergent fibre, ADF = acid detergent fibre, ADL = acid detergent lignin, SE = standard error, p = p-value at significance level $p < 0.050$.
[1]Baseline= mean baseline apparent nutrient digestibility/apparent mineral absorption (n = 8) in week 1
[2]Estimated marginal means of the treatment group= means per treatment over all repeated measures of week 2 to 5 adjusted for the baseline measurements in week 1 (inserted in the model as a covariate).

No effect of supplementation was observed on the plasma acyl carnitine profile, as an estimate of nutrient utilisation from enzymatic or fermentative origin ($p > 0.050$) (Table 4.5). Finally, trace element supplementation did not induce a difference in body weight (170 vs. 171 kg ± 3, $p = 0.875$).

Table 4.5. Trace element supplementation effects on estimated marginal means of plasma acyl carnitines concentrations in grass fed zebu (*Bos indicus*) bulls.

Parameter		Baseline[1]	Treatment		SE	p
			Control[2]	Supplement[2]		
Acyl carnitines (µmol/l)	Acetyl	6.9	5.5	5.3	0.3	0.670
	Propionyl	0.67	0.63	0.56	0.08	0.566
	Butyl	0.34	0.27	0.27	0.02	0.912
	Isovaleryl	0.09	0.11	0.12	0.01	0.325
	3-OH-butyryl	0.05	0.03	0.04	0.01	0.184
	3-OH-isovaleryl	0.07	0.05	0.06	0.007	0.557
	Methylmalonyl	0.05	0.03	0.04	0.004	0.776

[1]Baseline= mean baseline concentrations (n = 8) in week 1
[2]Estimated marginal means of the treatment group= means per treatment over all repeated measures of week 2 to 5 adjusted for the baseline measurements in week 1 (inserted in the model as a covariate).

4.4 Discussion

In the current study, trace element supplementation successfully raised plasma and faecal trace element concentrations of zebu bulls fed a local grass diet. This increase occurred despite natural variation in dietary trace elements, indicating a suboptimal supply for some elements (Cu, Se, I) while an adequate supply for others (Mn, Zn, Co) upon comparison with *B. taurus* requirements (NRC, 2000). Furthermore, plasma and faecal Cu concentrations were increased despite the presence of high concentrations of antagonists (Mo, S, Fe), capable to form insoluble complexes with Cu in the rumen. In excess, thiomolybdate complexes (formed by Mo and S), can also bind Cu at post absorption sites, additionally causing a thiomolybdate toxicity (Gould and Kendall, 2011).

Previous research indicated a Cu, Se and possibly Na deficiency problem in grazing zebu cattle in the study area (**Chapter 2**). In the current study, some animals had initial plasma mineral concentrations indicating a risk for trace element disorder according to Suttle (2010). Despite this, mean baseline and estimated marginal concentrations in both treatment groups were above these threshold values for *B. taurus* cattle. Insufficient research is available to determine whether these values are also applicable to *B. indicus*

cattle. Furthermore, despite the changed Cu and Zn plasma status in the supplemented group, activities of Cu and Zn-related enzymes ceruloplasmin and SOD did not differ significantly between groups, in contrast to earlier data (ceruloplasmin: Legleiter and Spears, 2007; SOD: Cao and Chen, 1991; Shaheen and Abd El-fattah, 1995), and ceruloplasmin activity at the onset and throughout the trial was above the threshold value suggested for *B. taurus* cattle by Laven *et al.* (2007). Consequently, although supplementation caused an improved status of the supplemented minerals, the status on itself was probably adequate for most bulls throughout the trial according to *B. taurus* threshold values. As the diet provided to the animals prior to purchase could have been of adequate mineral quality, the latter could be explained by the capacity of soft tissue storage for several minerals, e.g. in liver for Cu, thyroid for I, kidney for Se (NRC, 2005). Mineral analysis of several grass species in previous work around the region of origin of the animals does contradict the hypothesis of a correct diet prior to the trial: of 20 grass samples, 20 had copper concentrations below requirements for beef cattle (NRC, 2000) (range: 1.7-9.3 mg/kg DM), 12 for Zn (5.5-59 mg/kg DM) and 18 for Se (10-380 µg/kg DM) (**Chapter 1**). Another explanation for this phenomenon could be a difference between cattle species (*B. indicus* vs. *B. taurus*) in mineral metabolism resulting in different trace element requirements and normal plasma ranges. Earlier research detected such differences for plasma Cu concentrations within *B. taurus* (Mullis *et al.*, 2003; Ward *et al.*, 1995) as well as for Se (Rowntree *et al.*, 2004), therefore, similar or even larger differences could exist between these species as suggested by McDowell (1985).

Nevertheless, the main objective of the present study was to detect early effects of trace element supplementation on nutrient digestibility and utilisation. In the present study, trace element supplementation did not affect nutrient digestibility. The presence of the time × supplementation interaction for apparent DM, crude protein and ADL digestibility and apparent Mn and I absorption, originating from the major shift between the two groups in week 3 of supplementation cannot be attributed to a specific factor. However, the interaction between time and supplementation did not reflect a divergent difference between the two groups. Overall, it can be concluded that trace element supplementation did not improve apparent nutrient digestibility nor apparent mineral absorption in the present study, which is in accordance with the findings of

Grace *et al.* (2002) in horses. As an exception, the apparent absorption of Se and I was affected by supplementation. Both elements seemed to be absorbed to a higher degree with increasing dietary intake, which confirms the lack of homeostatically controlled absorption mechanisms in these elements (Suttle, 2010). For minerals, apparent absorption results should be carefully interpreted, as they merely provide an estimation of their bioavailability, since certain post-absorption processes and differences in excretion rates can result in differences in bioavailability despite equal apparent absorption rates (Ammerman *et al.*, 1995).

Finally, plasma acyl carnitines were not influenced by trace element supplementation. These circulating carnitine esters mirror the available citric acid cycle energy substrates and thus nutrient utilisation as described by Verbrugghe *et al.* (2009). Isovaleryl-, 3-OH-butyryl-, 3-OH-isovaleryl- as well as methylmalonylcarnitine are products of branched-chain amino acid catabolism (Michal, 1999) and represent the relative importance of amino acids as an energy source. Acetyl-, propionyl-, and butyrylcarnitine reflect the contribution of energy from the respective volatile fatty acids produced in fermentation (Bremer, 1983). The lack of effect of trace element supplementation on the presented products suggests that no alterations in energy metabolism were induced during the experimental period.

Conclusion

In the current study, concentrations of Mn, Zn, Cu and Se in plasma of grass-fed zebu (*B. indicus*) bulls were increased by trace element supplementation (Mn, Zn, Cu, Se, Co and I), indicating an altered mineral status although mean treatment values were still within normal ranges for *B. taurus* cattle. The latter is in sharp contrast to subnormal dietary Se and Cu concentrations according to *B. taurus* guidelines and could have implications for the applicability of *B. taurus* mineral requirements in *B. indicus* cattle. Ceruloplasmin and superoxide dismutase remained unaffected by supplementation. Supplementation of trace elements did not induce a difference in nutrient digestibility and utilisation, although it did affect apparent Se and I absorption.

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5

Impact of a trace element supplementation programme on health and performance of tropical crossbreed (*Bos indicus* × *Bos taurus*) dairy cattle: a double-blinded randomized field trial

Adapted from:

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Small-scale urban dairy farms (n = 16) in and around Jimma, Ethiopia with crossbred (Bos indicus × Bos taurus) cows were enrolled in a double-blinded intervention study to investigate the effect of a trace element supplementation programme on trace element status and milk concentrations as well as performance (body condition score (BCS), milk yield, leptin), milk composition, antioxidant status (ferric reducing ability of plasma (FRAP), thiobarbituric acid reactive substances (TBARS)), blood biochemistry, serum proteins and immune response (antibody titer upon rabies vaccination). The farms were allocated to a 1) placebo or 2) Cu, Zn, Se, Co and I supplementation treatment for 150d. On d 0 and d 120, four lactating cows per farm were sampled for milk and plasma, and on d 150 for serum, following primo-vaccination. Cu deficiency was present in 17% and marginal Se deficiency in 30 % of initially sampled cows, while no Zn shortage was detected. Over 120 days, trace element supplementation caused a bigger increase in plasma Se and Cu concentrations, but also a larger decrease of plasma Fe concentrations, as compared to the control group,. A larger increase in milk Se concentrations was observed in the supplemented group, whereas none of the other elements were affected. BCS decreased more over time in the supplemented group. None of the other parameters of performance and antioxidant status nor milk composition or blood biochemistry were affected by treatment. Antibody response to rabies vaccination did not differ between groups whereas α1- globulins tended to be lower and β-globulins tended to be higher in the supplemented group. In conclusion, despite improved Cu and Se status and Se concentrations in milk, cows on tropical urban dairy farms did not seem to benefit from trace element supplementation, with respect to the parameters investigated.

5.1 Introduction

The relationship between trace element status and a broad field of health conditions in dairy cattle has been clearly proven in industrialized regions (Enjalbert *et al.*, 2006; Guyot *et al.*, 2009), with shortages of Cu, Se and Zn known to be associated with retained placenta, impaired locomotion and diarrhoea (Enjalbert *et al.*, 2006). Therefore, trace element supplementation is a highly promoted practice, with reported positive effects, although most from experimental studies, on the incidence of mastitis (Machado *et al.*, 2013; Scaletti *et al.*, 2003), and immune response (Xin *et al.*, 1991).

In tropical regions, dairy cattle are most often the product of crossbreeding local types of zebu (*Bos indicus*) cattle with the temperate Holstein Friesian breed (*Bos taurus*), combining hardiness and productive characteristics (Fekadu *et al.*, 2011). Tropical urban dairy cattle are typically kept under challenging conditions, such as limited feed resources, high disease pressure and warm climate. Not surprisingly, these cattle are also prone to trace element deficiencies (Abdelrahman *et al.*, 1998; Belay *et al.*, 2009; Khalili *et al.*, 1993).

Unfortunately, trace element supplementation remains difficult to implement in developing countries, due to high costs and low accessibility. Moreover, trials examining the effect of mineral supplementation under tropical farming conditions are scarce. Investigating the effect of such supplements on a broad range of parameters, would clarify their efficiency under tropical conditions.

Our objective therefore, was to identify the effect of a trace element supplementation programme under tropical urban dairy farming conditions on trace element status, milk trace element concentrations, performance, milk composition, antioxidant status, blood biochemistry, serum protein electrophoresis and immune response to rabies vaccination, in a double-blinded randomized field trial.

5.2 Materials and methods

5.2.1. Farms and diets

In Jimma, South-West Ethiopia, severe bovine trace element deficiencies have been previously documented, with predominant shortages of Cu and Se (**Chapter 2**). Therefore, this region was selected as our study site. The local urban dairy cooperative, Jimma city multipurpose dairy development PLC, was approached and informed about our objectives. With their consent, we gathered information on dairy farms of members and selected urban (n = 16) dairy farms (Figure 5.1) with at least 4 lactating dairy cows to include in the trial.

Figure 5.1. Typical dairy farms enrolled in a double blinded intervention trial testing the effects of a trace element supplementation programme on health and performance of tropical crossbred (*Bos indicus* × *Bos taurus*) cows. **Left & Right**: farms have a low number of animals and poor housing conditions with a small surface area per cow.

On these farms, animals were housed in closed stables and were on a zero-grazing regime. Surface area per cow varied between 1.20 and 3.75 m^2. All animals included in the trial were Holstein Friesian (*Bos taurus*) × zebu (*Bos indicus*) crossbreeds, with the following characteristics, based on farmers' information, age: 6.8 ± 2.9 (SD) years, parity:

3.0 ± 1.7, month of lactation: 6.3 ± 3.8 and top milk yield: 13.5 ± 4.9 l/d. However, none of the farmers in the trial had a written account on age, parity, date of partus, milk yield or percentage of Holstein-Friesian. Yet, the farmers claimed to know this information by heart.

All animals received their regular diets with roughages and concentrates throughout the study. Feedstuffs dominating on the urban dairy farms consisted of concentrates and fresh grasses combined with some form of brewers' grains and bran (Table 5.1), with reported amounts of provided concentrates ranging between 4 and 6 kg, supplied with 2-5 kg of other grains and grasses/hays ad libitum.

Table 5.1. Characterization of urban dairy feeding management, Jimma, Ethiopia in terms of presence of absence

	Farm															
Ingredient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Concentrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Wheat bran	+		+		+	+			+			+	+		+	
Bean bran							+		+			+			+	
Rice bran													+			
Brewers grains[1]	+	+		+		+	+					+	+	+		
Atella[1]	+	+		+	+	+						+				
Oil seed cake													+			
Corn stem		+														
Enset[2]	+															
Hay		+													+	
Grass	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chicken feces												+				
Salt			+		+		+					+			+	
TEM	+	+	+	+	+	+	+	+								
Placebo									+	+	+	+	+	+	+	+

TEM = trace element mix

[1]Brewers grains= industrially processed brewers grains, atella= liquid by-product of local beer breweries (Solomon, 2007)

[2]Stem of *Ensete ventricosum*, false banana, widely cultivated as a food crop

However, farmers did not register diet composition or amounts provided and based on farmers' information, the feeding management could fluctuate within weeks based on local availability. No separate mineral supplements were utilized on the farms, except

for common table salt on five of sixteen farms. As an incentive for participating in the trial, all farmers received one kg of concentrate per dairy cow present at their farm.

5.2.2. Study design and treatment

Selected farms were stratified based on number of animals and gender of the farmer, to adjust for potential differences in management, such as smaller resources for investment and lower access to technical information in female farmers, but also better dairy performance when actively involved the implementation of new technologies (Mkenda-Mugittu, 2003; Mullins *et al.*, 1996). Afterwards, the farms were randomly assigned to a treatment: 1) placebo or 2) trace element supplementation of all adult cows present on farm, for 150 days. Placebo supplementation consisted of corn flour, supplied at a rate of 5 g/cow/d, providing 16 µg Cu/cow/d, 90 µg Zn/cow/d, 0.072 µg Se/cow/d, 1 µg Co/cow/day and 6 µg I/cow/day. Amounts of trace elements supplied were related to an estimated dry matter intake (DMI) of 10 kg/day, as 2.5% of an estimated body weight of 400 kg. Supplementation comprised of 250 mg Cu/cow/d, 540 mg Zn/cow/d, 3.0 mg Se/cow/d (organic, as chelated to glycine MAAC; Novus International Inc., MO) and inorganic 1.1 mg Co/cow/d ($CoSO_4.7H_20$) and 4.4 mg I/cow/d (NaI; Sigma-Aldrich, Bornem, Belgium).

Before the start of the trial, information sessions were organized to explain the function of trace elements and to elaborate on deficiency symptoms related to trace element shortages. Furthermore, the mode of treatment application, mixed in a spoon of molasses and top dressed on concentrate meal, was demonstrated. Supplements were distributed on a weekly basis and participation of farms was closely monitored by trial assistents, who reported an excellent consumption of supplements due to the high palatability of the molasses. Both farmers and trial assistants were blinded to treatment allocation to prevent bias.

5.2.3. Samples

On every farm, four adult lactating cows (at least 2 months in lactation) were randomly selected for sampling of plasma and milk, on d 0 (rainy season) and d 120 (dry season). All cows sampled on d 120 were vaccinated with inactivated rabies vaccine (Novibac Rabies, 1 ml IM, Intervet NV, Brussels, Belgium) and sampled for serum on d 150. The body condition score (BCS) of sampled animals (1-to-5 point scale) was noted on d 0 and d 120 by the same investigator (Wildman *et al.*, 1982).

Blood samples were obtained from the jugular vein using 18 G needles and two 9 ml sodium heparin tubes for plasma at d 0 and d 120, while two 6 ml cloth activator tubes (VWR International BVBA, Leuven, Belgium) were used for serum at d 150. Furthermore, a milk sample was obtained from each open quarter and equally pooled per cow at d 0 and d 120. Immediately after sampling, blood and milk samples were placed in ice water and within two hours transported to the laboratory. There, sodium heparin tubes were centrifuged at 1500 × *g* for 10 minutes after which plasma was separated, while cloth activator tubes were allowed to stand for 2 hours in the refrigerator, after which serum was separated through centrifugation at the same velocity. Milk samples were stored at 4 °C until milk composition analysis and California Mastitis Test (CMT) score determination. Aliquots of milk samples as well as plasma samples were stored at -20 °C until mineral analysis. Samples of the different feed stuffs, both roughages and concentrates, were collected at the onset of the trial. Feed stuff samples were oven-dried at 65 °C until constant weight and ground to pass a 2 mm screen.

5.2.4. Analytical methods

Milk samples were first analysed for composition by means of the Julie Z7 Automatic® analyser (Scope Electric, Regensburg, Germany), determining milk fat, protein, lactose, solids-non fat, solids and density. Also, the CMT was performed to detect and assess the degree of subclinical mastitis; the following scores were given to the pooled udder samples: 0 for no reaction, 1 for a trace, 2 for a weak positive, 3 for a distinct positive and 4 for a strong positive reaction (Bhutto *et al.*, 2012).

Feed, plasma and milk samples were analysed for mineral concentrations. Sample preparation for this analysis consisted of microwave digestion with 10 ml HNO_3 in closed vessels for feed samples while in open vessels for plasma and milk samples, both followed by filtration of the residue. Feed samples were analysed for Fe, S, Mo, Cu, Zn, Mn, Se, Co and I concentrations, while plasma and milk samples for Mo, S, Zn, Cu, Co, Se, Fe and Mn, using inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX radial, Varian, Palo Alto, CA) and inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, Perkin Elmer, Sunnyvale, CA). ICP-MS was used when results were observed to be below the detection limit of ICP-OES. These analytical procedures have previously proven to generate accurate data for feed, plasma and milk samples. Depending on the element and its concentration, recoveries of certified reference materials and standards spiked to the sample matrix varied between 85 and 110%, with most recoveries varying between 95 and 105%.

Levels of plasma leptin were determined using a multispecies radio immunoassay (RIA) kit (Millipore, St. Charles, MO) with guinea pig antihuman leptin as the antibody; levels are expressed as Human Equivalent (Cools *et al.*, 2010). Plasma lipid peroxidation, determined quantitatively as the thiobarbituric acid reactive species (TBARS) and expressed as malondialdehyde equivalents, was measured spectrophotometrically (Ultrospec III Pharmacia LKB Ltd., Cambridge, UK). The reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA) in the presence of added antioxidant (butylhydroxytoluene) was initiated in acidic conditions at 90°C. After quantitatively extracting the produced red chromogen with *n*-butanol:pyridine (15:1, v/v), the absorption at 532 nm in the extract was measured (Ohkawa *et al.*, 1979). For calibration and standardization, known concentrations of 1,1,3,3-tetramethoxypropane (TMP) were used under the very same test conditions, allowing expression of the oxidative status as MDA equivalents. The total antioxidant capacity of plasma, expressed as the ferric reducing ability of plasma (FRAP), was determined by the analytical procedure of Benzie & Strain (1996). The ferrous (Fe^{II}) ion concentration, produced by the reduction of the ferric (Fe^{III}) tripyridyltriazine complex by reducing plasma components, was determined spectrophotometrically. The absorbance at 593 nm was measured after a 5 minute

incubation of the mixture of the plasma sample and the reagent at 37°C; a calibration curve was established with standards of $FeSO_4$ allowed for quantification.

Activities of the plasma enzymes alkaline phosphatase (AP, EC 3.1.3.1), creatine kinase (CK, EC 2.7.3.2), γ-glutamyltransferase (GGT, EC 2.3.2.2), L-lactate dehydrogenase (LDH, EC 1.1.1.27), aspartate aminotransferase (AST, EC 2.6.1.1) as well as plasma total protein, urea and creatinin were determined spectrofotometrically using commercial kits (Abbott Laboratories, Abbott Park, IL, USA). The amount of serum albumine was determined using a colorimetric assay with bromocresol green, again using a commercial kit (Cobas, Roche Diagnostics GmbH, Mannheim) whereas serum proteins were differentiated with capillary ion electrophoresis. All tests were performed at a commercial laboratory (A-M-L, Antwerp, Belgium).

The neutralizing antibody titer against rabies virus was examined with the Rapid Fluorescent Focus Inhibition Test (RFFIT). The RFFIT was performed according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Office International des Epizooties (OIE), 2011). The neutralizing potency was expressed in International Units (IU)/ml in reference to "The Second International Standard for Anti-Rabies Immunoglobulin" purchased from the United Kingdom National Institute for Biological Standards and Control. According to the WHO convention, a serum titer of 0.50 IU/mL is protective *in vivo*.

5.2.5. Statistical analysis

All statistical analyses were performed using SAS Version 9.3 (SAS Institute Inc., Cary, NC). All response variables, with the exception of immune response and electrophoresis after primo-vaccination, were submitted to a mixed model with farm and cow nested in farm as random effects and sampling day, treatment and their interaction as categorical fixed effects. For each response variable, we tested whether the change over 120 days differed between the supplemented and control group, i.e. whether treatment and time interacted. For immune response and electrophoresis following primo-vaccination, a mixed model was used with treatment as fixed effect and farm as random effect, and the treatment effect was tested. Searching for association between plasma mineral

concentrations and other investigated parameters at the onset of the trial, Pearson correlation tests were used. Significance was declared at a probability level of $p < 0.050$.

5.3 Results

Mineral concentration in feed ingredients sampled at the urban dairy farms, are shown in Table 5.2. Feedstuffs were most commonly low in Cu and Se, based upon requirements for lactating Holstein cattle (Cu: < 11 mg/kg DM; Se: < 0.30 mg/kg DM; NRC (2001)), combined with high to even toxic concentrations of the Cu antagonist, Fe (> 1000 mg/kg DM; NRC (2005). Mo and S concentrations seemed below antagonistic ranges for Cu (Mo: < 1.5 mg/kg DM; S: < 0.2 mg/kg DM; Suttle (2010). Most feedstuff met requirements for other trace elements (Zn: > 43 mg/kg DM; Mn: > 14 mg/kg DM; Co: > 0.11 mg/kg DM; I: 0.60 mg/kg DM; NRC (2001).

Table 5.2. Mineral concentrations in feedstuff offered at urban dairy farms, Jimma, Ethiopia

	Zn	Cu	Se	Mn	Co	I	Fe	Mo	S
Ingredient	mg/kg DM								%
Concentrate	68	11	0.200	99	1.2	0.6	763	1.1	0.23
Wheat bran	87	11	0.245	125	0.06	0.3	118	1.2	0.16
Bean bran	39	3.5	0.083	43	0.97	0.7	414	0.84	0.04
Rice bran	32	5.2	0.110	139	0.65	0.1	713	0.73	0.10
Brewers grains[1]	151	22	0.145	64	0.90	2.9	625	1.6	0.25
Atella[1]	96	15	0.139	314	2.8	_	6486	_	0.21
Oil seed cake	69	27	0.198	284	7.6	1.5	10567	1.1	0.34
Corn stem	146	2.0	0.049	258	0.71	3.0	449	0.24	0.11
Enset[2]	10	3.1	0.084	1146	1.2	0.4	182	0.26	0.08
Hay (n = 2)[3]	33.5	6.55	0.098	501	1.74	1.26	778	0.72	0.08
	±2.12	±2.05	±0.007	±54	±0.39	±0.01	±411	±0.39	±0.02
Grass (n = 15)[3]	49	5.0	0.160	242	1.5	0.9	1811	1.2	0.20
	±20	±2.2	±0.090	±163	±0.7	±0.7	±1208	±0.8	±0.08
Chicken feces	224	25	0.552	224	2.62	2.9	2247	2.5	0.36
Salt	≤1.0	4.6	0.506	2	0.04	2.1	38	3.9	4.7

[1]Brewers grains= industrially processed brewers grains, atella= liquid by-product of local beer breweries (Solomon, 2007)

[2]Stem of *Ensete ventricosum*, false banana, widely cultivated in Ethiopia as a food crop

[3]Mean ± SD

Initial plasma Cu concentrations of 17% of animals were indicative of severe deficiency (< 0.57 mg/l; Suttle (2010)) whereas 28% was at least marginally Cu deficient upon comparison with threshold values for *Bos taurus* cattle (< 0.70 mg/l, Kincaid (2000). Another 30% of initially sampled cows were marginally Se deficient (< 0.060 mg/l; Puls (1988). However, no severe Se or Zn deficiency was observed (Se: < 0.030 mg/l; Puls (1988); Zn: < 0.58 mg/l; Suttle (2010)). Plasma Fe concentrations and levels of TBARS at the onset of the trial were significantly associated ($r = 0.57$, $p < 0.001$) (Figure 5.2).

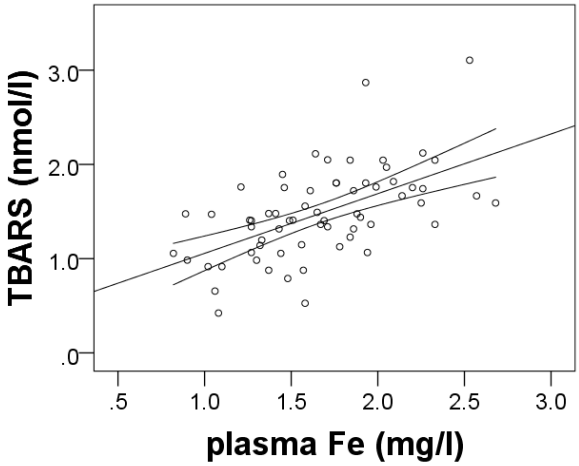

Figure 5.2. Association between plasma Fe and TBARS levels in crossbred (*Bos indicus* × *Bos taurus*) dairy cows at the onset of the trial. $r^2 = 0.323$, $p < 0.001$ Bent lines represent the 95% confidence interval of the mean, TBARS= thiobarbituric acid reactive substances

Over 120 d, cows in the trace element supplementation group experienced an increase in plasma concentrations of the supplemented Cu and Se, whereas plasma Cu concentrations diminished and plasma Se concentrations remained at the same level in the control group (Table 5.3) (both $p < 0.001$). Also, plasma concentrations of the Cu antagonist Fe decreased in the supplemented group, but increased in the control group ($p < 0.050$) (Table 5.3). On the contrary, plasma Mo concentrations tended to increase in the supplemented group ($p = 0.08$). None of the other minerals were affected by a time × treatment interaction (all $p > 0.050$). Overall, cows on d 120 had higher plasma concentrations of Zn, S, Se (all $p < 0.001$), and Mn ($p < 0.050$), but lower concentrations of Cu ($p < 0.010$) than cows sampled on d 0.

The positive change in milk concentrations of the supplemented Se over time was higher in the supplemented group (+0.025 vs. +0.009 mg/l; $p < 0.010$) (Table 5.4), but milk

concentrations of other minerals remained unaffected. On d 120, milk Cu ($p < 0.010$), Se ($p < 0.001$) and Co ($p < 0.050$) concentrations were higher than on d 0.

Furthermore, the supplemented group experienced a fall in BCS (Figure 5.3) over time whereas an increase was observed in the control group ($p = 0.001$). No differences in changes in other performance parameters between treatment groups were observed.

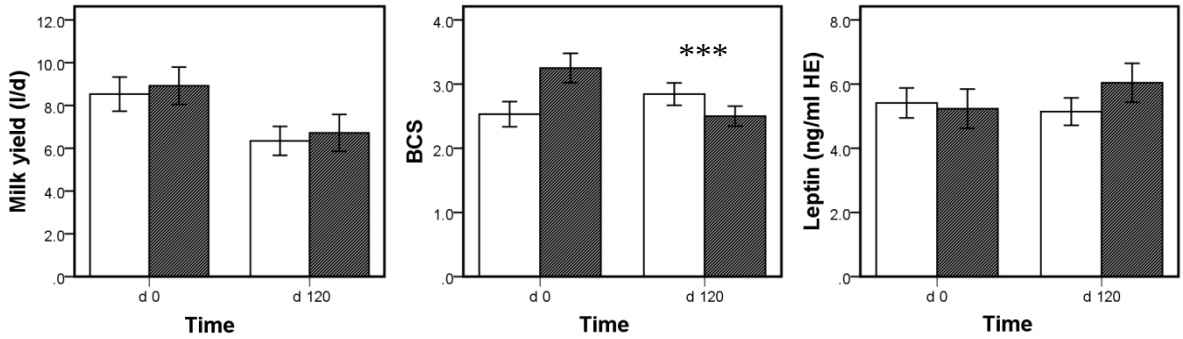

Figure 5.3. Effects of trace element supplementation on performance (milk yield, BCS, leptin ± SE) in crossbreed dairy cows. Light grey bars= placebo treatment, dark grey bars= supplement treatment, milk yield as perceived by farmers, BCS using a 1-to-5 scale (Wildman *et al.*, 1982). ***BCS: Significant time × treatment interaction ($p < 0.001$)

Equally, no differences in changes in milk composition and component yields (Table 5.5) between treatment groups were present. Reported milk yield and milk fat yield were lower on d 120 ($p < 0.050$) than on d 0, irrespective of treatment.

Changes in antioxidant status (Figure 5.4) and blood biochemistry (Table 5.6) did not differ significantly between treatment groups (all $p > 0.050$). TBARS values were lower ($p < 0.050$) on d 120, combined with higher CMT values ($p < 0.001$) than on d 0 (Figure 5.4). Plasma LDH, urea ($p < 0.001$), TA ($p < 0.010$), CK and total protein ($p < 0.050$) all were higher in cows sampled on d 120 compared to cows sampled on d 0 (Table 5.6). Average values of total protein and AST were above reference interval values provided by the laboratory (6.2-7.5 g/dl and 31-52 U/l respectively).

Table 5.3. Effects of trace element supplementation on plasma mineral concentrations in crossbreed dairy cows

	Treatment			
Parameter	Control	Supplemented	SEM	p-value[1]
Cu, mg/l			0.02	0.005
d 0	0.73	0.82		
d 120	0.59	0.83		
Zn, mg/l			0.03	0.728
d 0	1.02	0.95		
d 120	1.51	1.40		
Se, mg/l			0.004	<0.001
d 0	0.072	0.073		
d 120	0.075	0.143		
Co, mg/l			0.0002	0.415
d 0	0.0034	0.0026		
d 120	0.0034	0.0029		
Mn, mg/l			0.002	0.406
d 0	0.017	0.018		
d 120	0.028	0.023		
Mo, mg/l			0.001	0.081
d 0	0.035	0.033		
d 120	0.034	0.038		
S, mg/l			8	0.179
d 0	872	906		
d 120	941	1,008		
Fe, mg/l			0.04	0.036
d 0	1.60	1.73		
d 120	1.78	1.60		

[1]p-value for the time × treatment interaction

Table 5.4. Effects of trace element supplementation on milk mineral concentrations in crossbreed dairy cows

	Treatment			
Parameter	Control	Supplemented	SEM	p-value[1]
Cu, mg/l			0.003	0.886
d 0	0.077	0.097		
d 120	0.092	0.111		
Zn, mg/l			0.10	0.515
d 0	3.89	3.97		
d 120	4.09	3.96		
Se, mg/l			0.002	0.010
d 0	0.024	0.023		
d 120	0.031	0.048		
Co, mg/l			0.0001	0.979
d 0	0.0068	0.0074		
d 120	0.0083	0.0090		
Mn, mg/l			0.013	0.951
d 0	0.115	0.098		
d 120	0.117	0.098		
Mo, mg/l			0.002	0.836
d 0	0.059	0.064		
d 120	0.061	0.064		
S, mg/l			17	0.277
d 0	272	253		
d 120	367	274		
Fe, mg/l			0.02	0.099
d 0	0.57	0.62		
d 120	0.62	0.53		

[1]p-value for the time × treatment interaction

Table 5.5. Effects of trace element supplementation on milk composition in crossbreed dairy cows

	Treatment			
Parameter	Control	Supplemented	SEM	p-value[1]
Fat, %			0.20	0.220
d 0	4.36	5.33		
d 120	4.79	4.90		
Protein, %			0.04	0.206
d 0	2.65	2.72		
d 120	2.91	2.77		
Lactose, %			0.06	0.242
d 0	3.96	4.04		
d 120	4.34	4.13		
SNF, %			0.11	0.239
d 0	7.22	7.37		
d 120	7.91	7.53		
Fat yield, kg/d			0.08	0.359
d 0	0.36	0.51		
d 120	0.31	0.38		
Protein yield, kg/d			0.04	0.989
d 0	0.23	0.25		
d 120	0.20	0.22		
SNF yield, kg/d			0.10	0.994
d 0	0.62	0.67		
d 120	0.53	0.59		
Density, %			0.4	0.440
d 0	23.7	23.5		
d 120	25.9	24.4		
Solids, %			0.009	0.270
d 0	0.593	0.605		
d 120	0.650	0.620		

[1]p-value for the time × treatment interaction

Table 5.6. Effects of trace element supplementation on blood chemistry in crossbreed dairy cows

	Treatment			
Parameter	Control	Supplemented	SEM	p-value[1]
ALP[2], U/l			3	0.573
d 0	51	53		
d 120	52	58		
CK[3], U/l			4	0.387
d 0	104	108		
d 120	114	130		
GGT[4], U/l			1.0	0.346
d 0	26.6	30.9		
d 120	28.6	29.0		
LDH[5], U/l			18	0.317
d 0	814	853		
d 120	906	1,013		
Total protein, g/dl			0.08	0.276
d 0	7.98	7.94		
d 120	8.17	8.46		
AST[6], U/l			4	0.622
d 0	64	70		
d 120	82	95		
Urea, mg/dl			0.9	0.145
d 0	27.8	23.9		
d 120	31.6	31.5		
Creatinin, mg/dl			0.016	0.909
d 0	0.792	0.796		
d 120	0.832	0.831		

[1]p-value for the time × treatment interaction

[2]alkaline phosphatase activity

[3]creatine kinase activity

[4]γ-glutamyl transferase activity

[5]lactic dehydrogenase activity

[6]aspartate aminotransferase activity

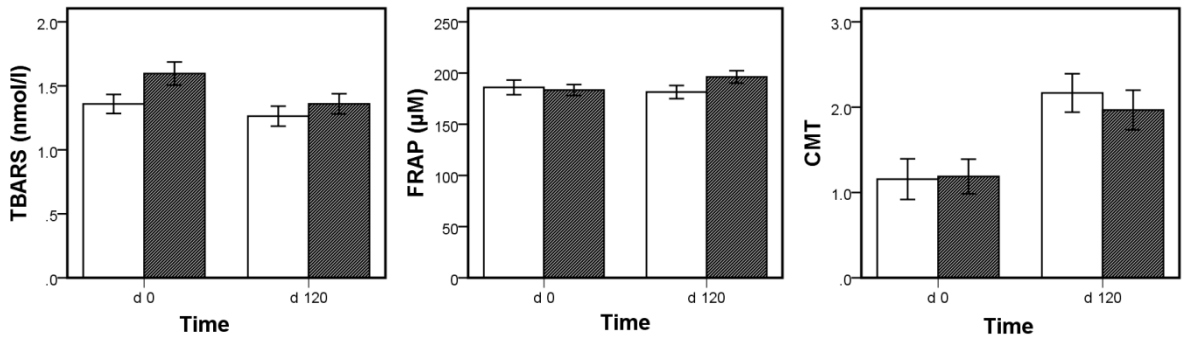

Figure 5.4. Effects of trace element supplementation on antioxidant status (TBARS, FRAP, CMT ± SE) in crossbreed dairy cows. Light grey bars= placebo treatment, dark grey bars= supplement treatment, TBARS= thiobarbituric acid reactive substances, FRAP= ferric reducing ability of plasma, CMT= California mastitis test. No significant time × treatment interactions (all p > 0.050).

Alpha1- and especially beta-globulins (Table 5.7) tended to differ between treatment groups (p = 0.09 and p = 0.05, resp.). Average beta-globulin percentages were slightly higher than the upper values from the reference interval provided by the laboratory (11.0-20.0 %). Antibody response to rabies vaccination was not affected by trace element supplementation (p > 0.050).

Table 5.7. Effects of trace element supplementation on serum protein differentiation and serum antibody titer in response to rabies vaccination in crossbreed dairy cows

	Treatment			
Parameter	Control	Supplemented	SEM	p-value
Albumine, %	36.5	36.0	0.6	0.792
α1-globulins, %	5.45	5.08	0.10	0.093
α2-globulins, %	12.8	12.3	0.2	0.375
β-globulins, %	21.1	23.8	0.6	0.054
γ-globulins, %	24.2	22.8	0.6	0.370
NAT[1], UI/ml	0.528	0.673	0.065	0.285

[1]NAT= rabies virus neutralizing antibody titer at 30 d after primovaccination (log-transformed)

5.4 Discussion

5.4.1. Feeds, plasma and milk trace element concentrations

In our study, although we had no data on total ration trace element concentrations, a vast majority of employed feedstuffs on tropical urban dairy farms provided Cu and Se concentrations below requirements, implying a high risk for Cu and Se deficiency. Furthermore, although, again, we could not calculate total dietary Cu:Mo and Fe:Cu ratio, the high Fe concentrations in most feedstuffs, can contribute to an even lower Cu absorption coefficient and therefore, to lower total amounts of available Cu (Suttle, 2010). On the contrary, Mo did not seem to playe an active role in the Cu antagonism. A substantial number of cows having plasma Cu concentrations below threshold values for *Bos taurus* cattle, at the onset and in the control group, did indicate that there was an inadequate Cu supply. The trace element supplementation programme successfully helped to maintain plasma Cu, but in general not to raise them majorly, despite the high Cu concentrations included in the supplement (250 mg/cow/day). This does indicate the presence of strong antagonists (see above). Furthermore, the strong association between plasma Fe and TBARS, only previously reported in rats (Linpisarn *et al.*, 1991), points to the negative effect of Fe on lipid peroxidation processes. A decrease in plasma Fe concentrations in the treatment group in contrast with an increase in the control group might contribute to the fact that supplementation indeed was a way to cope with the large Fe intake although we have no certainty on causality and this would contradict earlier research, as Hansen *et al.* (2010) mention that Cu deficiency leads to increased hepatic Fe storage and decreased plasma circulation, due to the lowered activity of ceruloplasmin. The trend towards a time × treatment interaction for plasma Mo concentrations ($p = 0.08$) with a seemingly larger increase in the supplemented group is difficult to interpret but represents a small difference and hence does not provide additional information on antagonism mechanisms in the sampled animals. In contrast to plasma Cu, concentrations of Se were greatly increased due to supplementation, as seen by Stowe and Herdt (1992), although none of the animals was severely Se deficient on d 0. Supplementation did not affect plasma Zn concentrations, which suggests an

adequate dietary supply and confirms the homeostatic mechanisms controlling the additional supply of Zn (Suttle, 2010).

Milk concentrations of Se were doubled by the supplementation programme which was in line with Ceballos *et al.* (2008), although the rise caused by organic Se supplementation was not as great as predicted by those authors (mean + 65 µg/l versus + 25 µg/l in our study) but equal to the rise observed by Juniper *et al.* (2006). Milk Cu and Zn concentrations on the contrary remained unaffected, confirming differences in secretion patterns between trace elements (Suttle, 2010), although Sobhanirad *et al.* (2010) did find increased milk Zn concentrations while supplementing 500 mg Zn/kg DM, levels much higher than offered in our study.

5.4.2. Performance and milk composition

Milk yield as estimated by the farmers was not impacted by the trace element supplementation, which is in line with earlier research (Cu: Engle *et al.* (2001); Se: Juniper *et al.* (2006); Zn: Sobhanirad *et al.* (2010)), although none of the farmers kept a written account of production parameters. Surprisingly, the supplemented group experienced a larger fall in BCS over time than the control group. The effect of supplementation on BCS during lactation has not been intensively studied before, although Sobhanirad *et al.* (2010) did not find an effect of Zn supplementation. Body condition score is affected by many herd related factors, such as stocking rate and level of concentrates fed to grazing cows as well as cow related factors, such as BCS at calving, parity and age, but also associated with productivity related parameters such as dry matter intake and milk production (Roche *et al.*, 2009). Given that parity and age were highly variable, the cause of this difference in BCS change is difficult to unravel and consequences related to this difference are not clear. Plasma concentrations of leptin, at least partially responsible for the regulation of food intake and adiposity of mammals, and assumed to decrease in Zn deficiency in rats (Mangian *et al.*, 1998; Kwun *et al.*, 2007), were not affected by trace element supplementation. Low leptin levels in dairy cattle are related with a negative energy balance and high milk yield (Liefers *et al.*, 2003). Consequently, the (insignificantly) higher increase in plasma leptin ($p = 0.15$) in the

supplemented cows in our study, does not seem to support the hypothesis concerning a higher milk yield in cows associated with a larger decrease in BCS. Milk composition was not impacted by trace element supplementation. For Zn, this is confirming data from Sobhanirad *et al.* (2010), while for Se with Juniper *et al.* (2006). For Cu, comparative data are absent. The meta-analysis of Rabiee *et al.* (2010) mentions higher milk fat and protein levels in cows supplemented with organic forms of Cu, Zn and Mn, however, they fail to mention whether this was in comparison with control or inorganic trace element supplemented groups. Overall, these trace elements do not seem to be limiting in milk composition processes. In general, the low milk protein does indicate a low energy supply (Coulon and Rémond, 1991), possibly hindering the full benefits of the trace element supplementation to the cows in the study.

5.4.3. Antioxidant status and blood biochemistry

Trace element supplementation did not significantly affect any of the parameters related to antioxidant status. For Se, this in contrast with Calamari *et al.* (2011), who found lowered TBARS values in heat-stressed cows supplemented with Se yeast versus control animals and Se selenate supplemented animals. Studies on the effect of Cu and Zn supplementation on the parameters studied in our work seem to be absent in literature. Summarized, organic Se supplementation might cause a change in the antioxidant status of dairy cattle according to earlier data, but this was not observed in our study. CMT scores were not affected by supplementation. In the study of Sobhanirad *et al.* (2010) organic Zn supplementation tended to induce a CMT difference in comparison with the control group, while for Se, Juniper *et al.* (2006) detected no differences. Blood biochemistry was not influenced by organic trace element supplementation, which is in line with Juniper *et al.* (2006). Overall, levels of AST and total serum protein were elevated, indicating chronic inflammatory or hepatic diseases (Russell and Roussel, 2007).

5.4.4. Serum protein electrophoresis and immune response after vaccination

The effect of organic micro mineral supplementation on adult dairy cow serum protein fractions has not been studied before. Mohri *et al.* (2005) suggested that vitamin E and Se supplementation raised IgM levels in treated calves, causing a higher β-globulin fraction. The trend towards a higher level of β-globulins ($p = 0.05$) in the supplemented group in our study could suggest either increased IgM or IgA levels in this group in response to the vaccination due to beta-gamma-bridging, either a higher degree of chronic infections and/or hepatic diseases in this group (Eckersall, 2008). Although not significantly affected by a time × treatment interaction, the relatively larger increase in serum total protein ($p = 0.28$) and AST levels ($p = 0.62$) may rather point to the second hypothesis. Thus, next to the negative effect on BCS, trace element supplementation may have negatively affected the presence of chronic inflammatory or hepatic diseases in the dairy crossbreeds in our study. Even though relatively higher, antibody response was not affected by trace element supplementation ($p = 0.29$). Comparative data in dairy cattle seem to be absent. For Zn, the lack of effect is in correspondence with data from Spears and Kegley (2002) investigating immune response to IBR vaccination in finishing beef steers fed organic and inorganic Zn versus control, but in contrast with differences in antibody response to *Brucella abortus* strains in crossbreed (*Bos indicus* × *Bos taurus*) bulls fed inorganic Zn versus control (Mandal *et al.*, 2007). For Cu, Ward *et al.* (1993) did not witness any difference (organic or inorganic versus control) in antibody titer against ovalbumin injection in growing beef steers, whereas for dairy cows, no data seem to exist. In a study investigating the effect of excess Se supplementation, treated growing beef cattle did not exhibit higher antibody titers against sheep red blood cells (Nicholson *et al.*, 1993). Consequently, trace element supplementation might affect antibody response to an antigen, but this was not seen in our study and contradictory data exist on the topic.

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6

Trace element distribution in selected edible tissues of zebu (*Bos indicus*) cattle slaughtered at Jimma, SW Ethiopia

Adapted from:

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The amount of trace elements present in edible bovine tissues is of importance for both animal health and human nutrition. This study presents data on trace element concentrations in semitendinosus and cardiac muscles, livers and kidneys of 60 zebu (Bos indicus) bulls, sampled at Jimma, Ethiopia. From 28 of these bulls, blood samples were also obtained. Deficient levels of Cu were found in plasma, livers, kidneys and semitendinosus muscles. Suboptimal Se concentrations were found in plasma and semitendinosus muscles. Semitendinosus muscle contained high Fe concentrations. Trace elements were mainly stored in the liver, except for Fe and Se. Cardiac muscles generally contained higher concentrations of trace elements than semitendinous muscles except for Zn. A strong association was found between liver and kidney concentrations of Cu, Fe, Co and Mo. Liver storage was well correlated with storage in semitendinosus muscle for Se and with cardiac muscle for Co and Se. Plasma concentrations of Cu, Se, Co were well related with their respective liver concentrations and for Co and Se, also with cardiac muscle concentrations. The data suggest multiple trace element deficiencies in zebu cattle in South-West Ethiopia, with lowered tissue concentrations as a consequence. Trace element distribution in B. indicus edible tissues seemed quite similar to Bos taurus distribution. However, tissue threshold values for deficiency in B. taurus cattle need to be refined and their applicability for B. indicus cattle needs to be evaluated.

6.1 Introduction

Deficiencies in trace elements, such as Se and Zn, are frequently observed in humans in tropical regions such as Ethiopia (Amare *et al.*, 2012), with severe health consequences (e.g. stunted growth, lowered antioxidant status) (World Health Organization, 1996). Meat and organ consumption form an important contribution to human nutrition, as these tissues have the capacity to store high amounts of trace elements (Berger, 2005). However, in Ethiopia, the world's fifth largest cattle holder (FAOSTAT, 2013), zebu (*Bos indicus*) cattle are typically free ranging on poor pastures and bovine trace element shortages (e.g. Cu deficiencies) are very common (**Chapter 1 & 2**). Unfortunately, data on trace element concentrations in edible tissues (such as meat, liver, kidney, heart) of *B. indicus* cattle and more specifically in Ethiopia are absent.

In *Bos taurus* cattle, the liver is considered the main indicator organ for status evaluation of several essential trace elements, assuming that it forms the main storage depot and is well related with storage in other tissues (Suttle, 2010). For at least some elements (e.g. Cu), especially at lower concentrations, a reasonable link of liver with plasma concentrations is present (Claypool *et al.*, 1975). A good relationship between liver and muscle Co and Zn concentrations was also found in earlier research (Blanco-Penedo *et al.*, 2010), whereas such a relationship was not noticed for other trace elements. The relationship between trace element concentrations in plasma and edible tissues (other than liver), however, was not studied before. The latter could be very important for human nutrition, as plasma concentrations might form a practical tool for early evaluation of trace element concentrations in meat, essential for optimal human health.

Furthermore, in *B. taurus* cattle, the distribution of trace elements over muscle tissues is still not well understood. Essential trace elements seem to distribute differently over different types of muscles (e.g. semitendinosus versus cardiac muscle) (García-Vaquero *et al.*, 2011), possibly related to muscle activity and fat content (Blanco-Penedo *et al.*, 2010). Furthermore, whether or not cattle with trace element deficiencies have a different trace element distribution pattern in edible tissues in comparison with cattle with an adequate status is not clear. Moreover, concentrations in *B. indicus* cattle may

differ intrinsically from those in *B. taurus* cattle. Therefore, the objective of the present study was to present tissue trace element distribution in Ethiopian zebu cattle and to evaluate the association of plasma and liver trace element concentrations with other tissue concentrations.

6.2 Materials and methods

6.2.1. Study area, animals and samples

The study was conducted in Jimma, the largest town in the Gilgel Gibe catchment area, Ethiopia, where bovine trace element deficiencies were previously recognized (**Chapter 2, 4** & **5**). At the local abattoir (Figure 6.1), receiving animals from the urban Jimma zone as well as from surrounding areas, adult zebu (*B. indicus*) bulls (n = 60) were randomly selected for sampling. Thereafter, 28 out of 60 bulls were randomly selected for immediate post-mortem blood sampling, using two sodium heparin tubes (VT-100SH, both Venoject®). Due to an inconsistent slaughtering scheme (Figure 6.1) it was impossible to sample both blood and tissues of all selected bulls.

Subsequently, from all 60 bulls, the cranial part of the left kidney, caudal lobe of the liver, semitendinosus and cardiac muscle (apex of the heart) were sampled. We greatly acknowledge Keraa abattoir and Jimma municipality for their kind permission to sample carcasses. Samples were immediately cooled and transported to the laboratory. Plasma was obtained through centrifugation at 1500 × *g* for 10 minutes and excessive fat was removed from tissue samples, where necessary. Samples were stored at -20°C until further analysis.

Figure 6.1. Keraa abattoir in Jimma, the sampling site of zebu bulls (n = 60) to investigate the trace element distribution in edible tissues.
Left: the main slaughter hall of the abattoir. **Right**: animal carcasses ready for transport to local restaurants and butcheries.

6.2.2. Mineral analyses

Muscle, kidney and liver samples were oven dried at 65°C until constant weight and ground through a 2-mm screen. Afterwards, samples were ashed through microwave destruction with 10 ml HNO_3 (Ultrapure analytical grade for trace element analysis) in open vessels followed by filtration. Finally, all samples were analysed for Zn, Cu, Fe, Se, Mo, Co and Mn concentrations through inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX radial, Varian, Palo Alto, USA) and inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, PerkinElmer, Sunnyvale, CA, USA). All glassware and microwave vessels were pre-rinsed with diluted HNO_3. A quality control program was employed throughout mineral analyses. Recovery rates from sampled matrices, spiked with two concentrations of the studied trace elements (in the range of the determined concentrations) were measured. Average recovery was 98%,

with a range between 82% (Zn in plasma) and 109% (Mo in kidney). Detection limits in acid digest were determined as: Mn 0.35 µg/l, Cu 0.25 µg/l, Mo 0.33 µg/l, Se 0.13 µg/l, Fe 21.4 µg/l, Zn 16.4 µg/l and Co 0.14 µg/l. Standards were run frequently alongside samples and all analytical results were blank-corrected.

6.2.3. Statistical analysis and reference value calculations

To detect whether differences were present for trace element concentrations between liver, kidney, semitendinosus and cardiac muscle, tissue concentrations were compared using a signed rank test at the 5% significance level with Bonferroni's adjustment technique for pairwise comparisons. Median and the first (Q1) and third (Q3) quartiles are reported. Spearman rank correlation coefficients (r) were used to determine the association between liver and plasma and other tissue concentrations of trace elements. Diagnostic threshold concentrations for *B. taurus* cattle stated in literature (Puls, 1988) and (Suttle, 2010) are expressed on wet weight (WW) basis, and in order to compare with the current data, were recalculated to dry weight (DW) basis by multiplying with the specific conversion factors stated by these authors: 3.5 for liver, 4.5 for other tissues (Puls, 1988); 3.3 for liver respectively (Suttle, 2010).

6.3 Results and discussion

To the best of our knowledge, this is the first study presenting data on trace element concentrations in edible tissues in zebu (*B. indicus*) cattle. However, our data also suggest the clear and urgent need for refinement of bovine plasma and liver thresholds of deficiency, as a practical reference to evaluate the need for supplementation. Discrepancies were present upon evaluation of plasma and liver concentrations based on diagnostic criteria for deficiency in *B. taurus* cattle.

Upon comparison of liver concentrations with these diagnostic criteria, 42 % of animals (n = 60) were considered severely Cu deficient (< 19 mg/kg DW; Suttle, 2010) (Table 6.1).

Plasma Cu concentrations in 29% of animals (n = 28) reflected this deficiency (< 0.57 mg/l; Suttle, 2010) (Table 6.2). Furthermore, semitendinosus muscle and kidney Cu concentrations were below concentrations considered adequate in *B. taurus* cattle in 97% and 100% of animals (n = 60; < 5.4 mg/kg DW; <18 mg/kg DW respectively; (Puls, 1988)). Therefore, bovine Cu deficiency in this region, as found in previous research (**Chapter 2, 4 & 5**) was confirmed by both plasma and liver concentrations.

On the contrary, it was not clear whether or not a Mn, Fe or Se deficiency was present in the area due to conflicting interpretations based upon plasma and liver concentrations (Puls, 1988; Suttle, 2010), as well as a wide range in threshold values found in literature, mentioned earlier (**Chapter 2**). For instance, liver samples did not reveal deficiencies for Fe, Se (n = 60; all but one > 150, all > 0.07 mg/kg DW respectively; Suttle, 2010) or Co, Zn and Mn (all > 0.018, > 70 and > 3.5 mg/kg DW respectively, Puls, 1988). On the contrary, plasma concentrations did indicate a severe Mn and Fe deficiency (< 0.02 mg/l and < 1.0 mg/l respectively, Suttle, 2010) in 29% and 11% of animals (n = 28) respectively. For Se, although none of the animals had plasma concentrations below diagnostic thresholds for deficiency according to Suttle (2010), 82% of animals (n = 28) had plasma Se concentrations considered at least marginally deficient (< 0.06 mg/l), according to Puls (1988). In this respect, especially threshold values for Se and Mn both in liver and plasma vary largely among authors (Kincaid, 2000; Puls, 1988; Suttle, 2010).

When comparing kidney and muscle concentrations of other trace elements with adequate ranges for *B. taurus* cattle, most strikingly, semitendinosus muscle Fe concentrations registered above this range in 73% of animals (n = 60; 80 > 54 mg/kg DW; Puls, 1988), whereas 60% of animals (n = 60) had semitendinosus muscle Se concentrations below the adequate range (< 0.32 mg/kg DW, Puls, 1988). It remains unclear whether or not the mentioned thresholds values and adequate ranges are to be extrapolated from *B. taurus* to *B. indicus* cattle as described in **Chapter 4**, seeing that even within *B. taurus* cattle, differences in breed sensitivity to deficiency are present (Mullis *et al.*, 2003; Ward *et al.*, 1995). Detailed studies at farm level investigating differences between *B. taurus* and *B. indicus* cattle in response variables (e. g. mRNA expression of trace element related genes) to a depletion diet and repletion through supplementation are necessary to clarify this.

Table 6.1. Trace element concentrations (mg/kg DW) in zebu (*Bos indicus*) bull (n = 60) tissues sampled at Jimma, Ethiopia with median, first quartile (Q1) and third quartile (Q3) as summary statistics

	Liver			Kidney			Muscle					
							Semitendinosus			Cardiac		
Element	Median	Q1 - Q3	Adequate[1]	Median	Q1 - Q3	Adequate[1]	Median	Q1 - Q3	Median	Q1 - Q3	Adequate[1]	
Cu	28.3[a]	7.6 - 65.6	88 - 350	13[b]	12 - 16	18 - 27	2.9[d]	2.0 - 3.6	16[c]	15 - 17	5.4 - 6.8	
Fe	306[a]	245 - 415	158 - 1050	369[a]	286 - 503	135 - 675	80[c]	53 - 105	210[b]	194 - 239	45 - 54	
Mn	13[a]	11 - 14	8.8 - 21	5.2[b]	4.6 - 5.6	5.4 - 9.0	1.1[d]	0.8 - 1.8	2.4[c]	1.9 - 3.0	2.0 - 3.8	
Zn	152[a]	121 - 201	88 - 350	107[b]	94.5 - 126	81 - 113	103[b]	83 - 148	81[c]	75 - 85	-	
Co	0.47[a]	0.38 - 0.65	0.07 - 0.30	0.39[b]	0.25 - 0.59	-	ND[d]	ND - ND	0.23[c]	0.14 - 0.37	-	
Mo	3.8[a]	3.3 - 4.2	0.49 - 4.9	1.9[b]	1.8 - 2.2	1.0 - 2.6	ND[d]	ND - 0.32	0.33[c]	0.26 - 0.42	-	
Se	0.76[b]	0.65 - 0.88	0.88 - 1.8	4.8[a]	4.3 - 5.3	4.5 - 6.8	0.37[d]	0.23 - 0.49	0.74[c]	0.59 - 0.86	0.32 - 0.68	

DW = dry weight

[1]Adequate range for cattle (Puls, 1988)

[a,b]Medians sharing a same letter do not differ significantly from each other ($p < 0.050$)

An additional hurdle when comparing tissue concentrations with threshold values or other comparative data is the wide variability in dry matter content, both between and within tissues. Dry matter concentrations in our study averaged 29% (range: 23-35%) for liver samples, 24% (17-42%) for kidney, 23% (15-31%) for semitendinosus and 22% (18-27%) for cardiac muscle samples. Because of this, if stated concentrations are expressed in a different unit, e.g. on fresh matter basis, conversion to this unit using a single conversion factor, might create bias. We therefore recommend authors stating data and diagnostic threshold concentrations, to at least mention average dry matter concentrations per tissue.

Table 6.2. Trace element concentrations in zebu (Bos indicus) bull (n = 28) plasma sampled at Jimma, Ethiopia with median, first quartile (Q1) and third quartile (Q3) as summary statistics

Mineral	Median	Q1	-	Q3	Adequate[1]			Threshold value[2]
Cu, mg/l	0.7	0.5	-	0.8	0.8	-	1.5	0.6
Fe, mg/l	1.7	1.2	-	2.0	1.3	-	2.5	1.0
Mn, µg/l	45	18	-	60	6	-	70	20
Zn, mg/l	1.2	1.1	-	1.3	0.8	-	1.4	0.6
Co, µg/l	3.9	2.8	-	5.2		-		0.9[a]
Mo, µg/l	26	19	-	34	10	-	50	100[b]
Se, µg/l	45	36	-	54	80	-	300	20

[1]Adequate range for cattle (Puls, 1988)
[2]upper threshold value indicating a deficiency risk in *Bos taurus* cattle (Suttle, 2010)
[a]Co: lower boundary of normal Co concentrations in *B. taurus* cattle (Puls, 1988),
[b]Mo: lower boundary of Mo concentrations in *B. taurus* cattle considered elevated (Puls, 1988)

In zebu (*B. indicus*) bulls sampled at Jimma, Ethiopia, liver contained the highest concentrations of trace elements compared to kidney, cardiac and semitendinosus muscle (Table 6.1), except for Se, of which concentrations were highest in kidney (all $p < 0.010$), and for Fe, for which we found no difference between liver and kidney concentrations ($p = 0.035$). Despite significantly higher Se concentrations in the kidney, the liver may still function as a main storage tissue for Se due its larger weight, in agreement with previous research (Herdt & Hoff, 2011). Fe seemed evenly distributed over liver and kidney, but the tissue weight may again result in the liver being the main storage entity. Liver contained the highest Zn concentrations, but Zn concentrations did not seem to vary widely among liver, kidney and muscle as observed earlier (López Alonso *et al.*, 2000).

Between muscle types, cardiac muscles systematically contained higher concentrations of trace elements than semitendinosus muscles, except for Zn of which concentrations were lower in the cardiac muscle samples (all $p < 0.001$, for Mo: $p = 0.001$), hence, demonstrating a profound difference in micromineral profile between muscle types. Comparative data in *B. indicus* cattle are absent, and data in *B. taurus* cattle are rare since sampled muscles are often not specified. However, our findings are generally in agreement with earlier research in *B. taurus* cattle (García-Vaquero *et al.*, 2011). Molybdenum formed an exception, considering the higher concentrations in cardiac muscle as compared to semitendinosus muscle in the current study, which contradicts the earlier data (García-Vaquero *et al.*, 2011). This may be explained by higher Mo concentrations in the environment and forages in the region (**Chapter 1 & 4**) leading to a higher accumulation in the *B. indicus* cattle in general and in cardiac muscle more specifically, although none of the sampled tissues contained Mo concentrations beyond the normal ranges stated for *B. taurus* cattle (Puls, 1988).

Focussing on the relation between concentrations of trace elements in liver as main storage organ and the other edible tissues sampled in the current study, we found a strong association between liver and kidney concentrations of Cu, Fe, and Co ($r = 0.53$, $r = 0.65$, $r = 0.80$ respectively; all $p < 0.001$) (Table 6.3), whereas liver and kidney concentrations of Mn, Zn and Mo were weakly correlated ($r = 0.28$, $p = 0.03$; $r = 0.38$, $p = 0.003$; $r = 0.39$, $p = 0.002$ respectively). To conclude, for most trace elements, there was a reasonable association between these two tissues, which contradicts previous research (López Alonso *et al.*, 2004).

There was a strong relation between liver and semitendinosus muscle concentrations of Se ($r = 0.57$, $p < 0.001$) and a weak correlation for Mn ($r = 0.36$, $p = 0.005$) whereas for other elements, no significant association were found between these two tissues. Cardiac muscle concentrations of Co and Se were strongly correlated with liver concentrations of the same elements ($r = 0.71$, $r = 0.75$, respectively; both $p < 0.001$). Additionally, there was a weak positive association between liver and cardiac concentrations of Fe and Mo ($r = 0.28$, $p = 0.03$; $r = 0.37$, $p = 0.003$) and a weak negative association for Zn ($r = -0.34$; $p = 0.007$). Blanco-Penedo *et al.* (2010) earlier found a strong association for Co and Zn between liver and diaphragm muscle, but not for Se, the latter

being in constrast to our study. Overall, in the present study, liver status seemed to correlate reasonably well with storage in other tissues, especially kidney and cardiac muscle.

Table 6.3. Spearman rank correlation coefficient between liver and other tissue concentrations of trace elements in zebu (*Bos indicus*) bulls (n = 60) at Jimma, Ethiopia

Element	Plasma vs. liver	Liver vs.			Plasma vs.		
		Kidney	Muscle		Kidney	Muscle	
			Semitendinosus	Cardiac		Semitendinosus	Cardiac
Cu	0.68***	0.53***	0.01	0.08	0.25	-0.14	0.16
Fe	-0.06	0.65***	0.19	0.28*	0.29	0.11	0.19
Mn	0.42*	0.28*	0.36**	0.19	0.22	0.37	0.16
Zn	-0.39*	0.38**	-0.24	-0.34**	-0.32	0.08	0.18
Co	0.61***	0.80***	0.14	0.71***	0.82***	0.24	0.69***
Mo	0.25	0.39**	0.05	0.37**	-0.05	-0.31	-0.21
Se	0.74***	0.17	0.58**	0.75***	0.15	0.71***	0.83***

*p < 0.050, **p < 0.010, ***p < 0.001

Plasma is often presented as a practical sample to assess trace element status (Herdt & Hoff, 2011). In the current study, plasma concentrations of Se, Cu, Co were strongly associated with liver concentrations of the same elements (r = 0.74, r = 0.68, r = 0.61; all p < 0.001) whereas for Mn and Zn, only a weak relation was present, which was even negative for Zn (r = 0.42, r = -0.39; both p < 0.050). For kidney, only Co concentrations were significantly associated with plasma concentrations (r = 0.82, p < 0.001). Furthermore, only Se semitendinosus muscle concentrations were associated with plasma concentrations (r = 0.71; p < 0.001). Finally, Se and Co cardiac muscle concentrations were strongly related with plasma Se and Co concentrations (r = 0.83, r = 0.69; both p < 0.001). Overall, plasma Cu, Co and Se seemed to associate well with their respective liver concentrations and reasonably well for Mn. This was not found for Fe and Mo or even negative Zn.

To conclude, although based on a small sample size, our data suggest that plasma Co and Se are probably very suitable for evaluation of liver status whereas Cu concentrations were well associated but should probably only be used in the case of expected low liver Cu concentrations (Claypool *et al.*, 1975). In this regard, the current results discourage

the use of plasma Mo, Zn, and especially Fe concentrations, although mentioned previously (Herdt & Hoff, 2011). Finally, plasma concentrations of Co and Se were well related with cardiac muscle concentrations.

Overall, our results confirm the differential distribution patterns of Se, Co vs. the other elements, explained by different sites of homeostatic control mechanisms. Homeostatic control of Se and Co is mediated by renal excretion, causing a continuing rise in storage related with rising dietary intake. For the other elements, the intestine is the site of homeostatic control, and consequently, tissue storage might plateau when requirements are met (Windisch & Ettfie, 2008). In general, our data also point to the liver as a better indicator sample than plasma for investigating trace element storage in cattle due to the higher number of significant correlations with other tissues.

Conclusion

Zebu cattle seemed to have a similar distribution of trace elements in edible tissues as *B. taurus* cattle. Different distribution patterns in edible tissues of Cu, Zn, Mn, Mo and Fe versus Co and Se were observed. Within the ranges observed in our study, plasma values were well related with liver status of Cu, Se and Co and even with muscle Se and Co concentrations. However, this study did confirm the liver as the main indicator organ to investigate bovine trace element status, as for most elements, it correlated well with other tissues.

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7

A disparate trace element metabolism in zebu (*Bos indicus*) and crossbred (*Bos indicus* × *Bos taurus*) cattle in response to a Cu deficient diet

Adapted from:

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Copper deficiency is a commonly diagnosed problem in cattle around the globe. In Jimma, Ethiopia, 8 zebu (Bos indicus) and 8 zebu × Holstein Friesian cross (Bos taurus × Bos indicus) heifers were used in an 11 weeks study, to investigate breed type differences in effects of Cu deficiency on concentrations of trace elements in plasma and edible tissues as well as mRNA expression of Cu-related proteins. Heifers were fed a grass diet (6.4 ± 0.7 (SD) mg Cu/kg DM), supplemented with 1 mg Mo/kg DM in week 1 to 4, and 2 mg Mo/kg DM in week 5 to 11, with blood samples collected every 2 week and tissue collection post-mortem. Plasma, liver, kidney, semitendinosus, and cardiac muscle were analysed for Zn, Cu, Fe, Se, Mo, Co, and Mn. Expression of mRNA Cu-related proteins was measured in aorta (lysyl oxidase, LOX), liver (Cu transporting β-polypeptide, Atp7b; Cu chaperone for superoxide dismutase, CCS; cytochrome c oxidase assembly homolog 17, Cox17; Cu transporter 1 homolog, Ctr1; superoxide dismutase 1, Sod1), and duodenum (diamine oxidase, DAO, metallo-thionein-1A, Mt1a) as well as the Se-related glutathione peroxidase 1 (Gpx1). Zebu cattle maintained initial plasma Cu concentrations just below the threshold value for deficiency, whereas crossbred cattle gradually became severely Cu deficient over time ($p < 0.001$). In contrast, plasma Zn and Co were greater in zebu cattle at the onset of the trial but became similar to crossbred cattle towards the end of the trial ($p < 0.001$). Liver Cu ($p = 0.002$) and Fe ($p = 0.0004$), kidney Se ($p < 0.0001$), and kidney and cardiac muscle Co ($p \leq 0.0001$) concentrations were significantly greater in zebu than in crossbred cattle. Increased hepatic mRNA expression of the Cu-regulatory proteins Atp7b, Ctr1 (both $p = 0.02$), CCS ($p = 0.03$), Cox17 ($p = 0.009$), and Cu-related enzyme Sod1 ($p = 0.001$) as well as the Se related Gpx1 ($p = 0.0002$) were significantly greater in zebu than in crossbred cattle. However, duodenal mRNA expression of DAO ($p = 0.8$) and Mt1a ($p = 0.2$), and aortic expression of LOX ($p = 0.8$) were not significantly different. Both the differences in Cu status indices (plasma and liver concentrations) and hepatic mRNA expression of Cu regulatory proteins point to the possibility of a more efficient use of dietary Cu in B. indicus as compared to Bos taurus × Bos indicus cattle resulting in greater sensitivity to Cu deficiency in Bos taurus crossbred cattle.

7.1 Introduction

The dietary micromineral supply for cattle is often low in tropical countries (McDowell & Arthington, 2005). Mineral imbalances in the soil and consequently in the plants growing on this soil, often aggravate the situation (Haque *et al.,* 1993). In Ethiopia, Cu deficiency is a frequent problem when grazing on pastures, as low levels of Cu are accompanied by high levels of Mo and S and very high levels of Fe (**Chapter 1** & **4**; Faye *et al.,* 1991; Faye & Grillet, 1984; Roeder, 1980). Consequently, deficiencies of Cu are to be expected in cattle in these regions.

Zebu cattle (*Bos indicus*) are the most commonly used type of cattle in Ethiopia, and renowned for their robustness under tough conditions (Edea *et al.,* 2013). Moreover, they seemed to have adapted to their environment very well, with reports indicating an ability to cope with dietary antagonists, such as tannins (Yisehak *et al.,* 2012). However, because of their low production capacity, crossbreeding these *B. indicus* cattle with *Bos taurus* types, is becoming popular (Fekadu *et al.,* 2011). Within *B. taurus* cattle, breed related sensitivity to Cu deficiency has been documented, with the Simmental breed reported as being most vulnerable (Mullis *et al.,* 2003; Ward *et al.,* 1995).

Several studies suggest that there may be a difference between *B. taurus*-influenced and *B. indicus* cattle in their ability to cope with an inadequate Cu supply. Frequently no signs of clinical Cu deficiency are seen in studies with zebu cattle despite a severely inadequate Cu supply based upon recommended levels for *B. taurus* cattle (**Chapter 2**; Faye & Grillet, 1984; Roeder, 1980). Other studies suggest that there might be a difference in response to the same diet between the local zebu and *B. taurus* crossbred cattle (Abu Damir *et al.*, 1988; Friot, 1973).

As we hypothesized that *B. indicus* may have adapted to a low Cu intake, hence optimising the use of Cu in their metabolism, our aim was to evaluate the effect of a Cu deficient diet on concentrations of Cu and other trace elements in plasma and selected edible tissues as well as mRNA expression of Cu-related proteins in *B. indicus* and *B. taurus* × *B. indicus* cattle.

7.2 Materials and methods

7.2.1. Animal care and experimental design

Sixteen *B. indicus* (Abyssinian Highland zebu; n = 8) heifers and *B. taurus* × *B. indicus* crossbred (Holstein Friesian × Abyssinian Highland zebu; n = 8) heifers with an average age of one year and six months (SD: 0.4 years) were purchased and housed at the dairy farm of the Jimma University Campus of Agriculture and Veterinary Medicine, Ethiopia. In the surrounding area, bovine Cu deficiency was seen on multiple occasions and Se deficiency was suspected based upon tissue levels below threshold values for *Bos taurus* cattle (**Chapter 2, 4, 5** & **6**).

Initial BW was lower in zebu than in crosses (91 ± 5.3 vs. 139 ± 12.9 kg, p = 0.004). Zebu cattle had an average BCS of 5.0 on a scale of 1 to 9 (Nicholson & Butterworth, 1986), whereas crosses had an average BCS of 3.4 ± 0.23 on a scale of 1 to 5 for *B. taurus* dairy cattle (Wildman *et al.*, 1982). Zebu BCS were recalculated to a scale of 1 to 5 afterwards, for comparison purposes (mean: 2.8 ± 0.25, type difference: p = 0.1).

All animals were individually housed and fed with a forage-only diet, consisting of local grasses, freshly harvested and chopped, during 11 weeks. This diet contained 6.4 ± 0.2 mg Cu/kg DM, 2.1 ± 0.4 mg Mo/kg DM and 2.4 ± 0.12 g S/DM (Table 7.1). Daily amounts of grasses provided were set to provide an DM intake of 2 % of BW (McDowell, 1996). Refusals were weighed and subtracted from the recorded DMI.

To induce Cu deficiency (Figure 7.1), Mo (as $NaMoO_4$, Sigma-Aldrich, St Louis, MO) was supplemented at 1 mg Mo/kg DM during week 1 to 4 and was afterwards raised to 2 mg Mo/kg DM from week 5 to week 11. The supplements were mixed in a spoon of molasses and top-dressed on the grass diet.

Table 7.1. Chemical composition of basal grass diet (n = 11).

Component	Mean	SD
CP, % DM	11.5	1.23
ADF, % DM	35.4	2.69
NDF, % DM	67.7	1.96
S, g/kg DM	2.4	0.03
Fe, mg/kg DM	250	93
Mn, mg/kg DM	102	53
Zn, mg/kg DM	64	13
Cu, mg/kg DM	6.4	0.7
Co, mg/kg DM	0.22	0.07
Mo, mg/kg DM[1]	2.1	1.3
Se, mg/kg DM	0.11	0.10

SD = standard deviation, [1]Supplemented with 1.0 mg Mo/kg DM from week 1 to 4, with 2.0 mg Mo/kg DM from week 5 to 11, as $NaMoO_4$

7.2.2. Samples and storage

Representative feed samples were obtained and pooled for analysis 3 times a week. Every 2 weeks, 9 ml blood was collected from all animals by jugular venipuncture using 18 G needles (450069) and 2 sodium heparin tubes (455051, both Vacuette®, Greiner Labortechnik, Austria).

At week 11, all animals were brought to a local slaughterhouse and slaughtered. Approximately 50 g of liver (caudal lobe), kidney (cranial part of left kidney), cardiac (heart apex) and semitendinosus muscle were collected to determine trace element concentrations in these tissues (Figure 7.1). Additionally, samples of aorta, small intestine and liver were collected to determine mRNA expression of Cu transporters and Cu related enzymes.

Plasma was obtained through centrifugation at 1500 × *g* for 10 minutes at 25 °Cand was stored at -20°C until further analysis. Grass samples as well as liver, kidney, cardiac and semitendinosus muscle samples were initially frozen at – 40 °C, afterwards oven dried at 65°C until constant weight and ground through 2 mm sieve. Tissue samples for mRNA analyses were immediately immersed in 10 ml RNAlater (Sigma-Aldirch, St Louis, MO) and frozen at -40 °C.

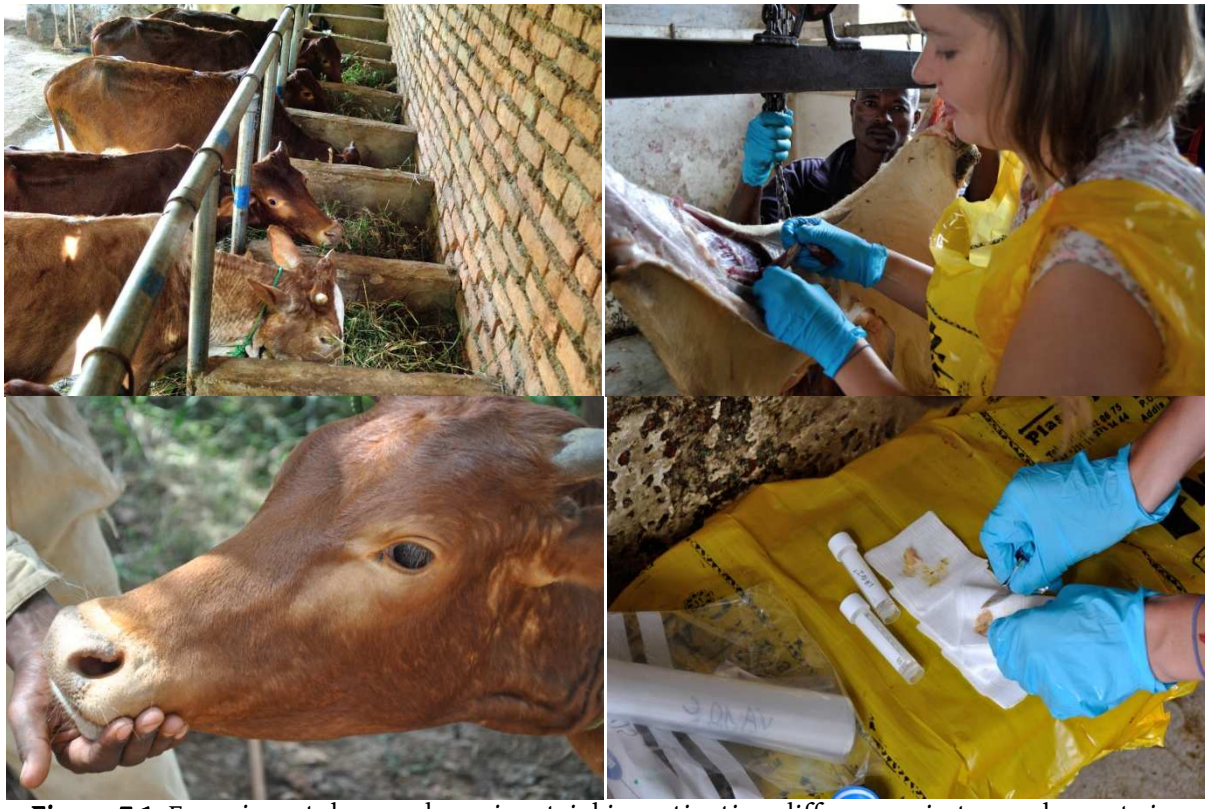

Figure 7.1. Experimental procedures in a trial investigating differences in trace elements in zebu and crossbred heifers fed a Cu deficient diet. **Upper left**: heifers ingesting the Cu deficient diet. **Lower left**: a close-up of one heifers exhibiting depigmentation of hairs around the eyes, a typical sign of Cu deficiency (called "copper glasses"). **Upper right**: sampling muscle. **Lower right**: obtaining aliquot of intestinal sample for mRNA analysis.

7.2.3. Analytical procedures

Grass samples were analysed for crude protein (CP) (984.13) by means of proximate analysis (AOAC, 1996). Additionally, acid detergent fibre (ADF) was analysed according to AOAC (1996); 973.18 and neutral detergent fibre (NDF) by a method of Van Soest (1991). Plasma, grasses and tissue samples were prepared for mineral analysis through microwave destruction with 10 ml HNO_3 (Ultrapure analytical grade for trace element analysis) in open vessels followed by filtration. All samples were analysed for Zn, Cu, Fe, Se, Mo, Co and Mn concentrations through inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX radial, Varian, Palo Alto, USA) and inductively coupled plasma mass spectrometry (ICP-MS) (Elan DRC-e, PerkinElmer, Sunnyvale, CA, USA). All glassware and microwave vessels were pre-rinsed with diluted HNO_3. A quality

control program was employed throughout mineral analyses. Recovery rates from sampled matrices, spiked with two concentrations of the studied trace elements (in the range of the determined concentrations), were measured. Average recovery was 98%, with a range between 82% (Zn in plasma) and 109% (Mo in kidney). Detection limits in acid digest were determined as: Mn 0.35 µg/l, Cu 0.25 µg/l, Mo 0.33 µg/l, Se 0.13 µg/l, Fe 21.4 µg/l, Zn 16.4 µg/l and Co 0.14 µg/l. Standards were run frequently alongside samples and all analytical results were blank-corrected.

7.2.4. Quantitative Real Time PCR

Total RNA was isolated from tissue samples using either the (intestine and liver) RNeasy Mini Kit or (aorta and skin) RNeasy Fibrous Tissue Mini Kit (both QIAGEN, Manchester, UK). Tissue samples (mean: 48 mg, range: 35-60 mg) were homogenised in a TissueLyser (Qiagen), using 500 µl of lysis buffer from the extraction kits and a 5 mm steel ball bearing in a 2 ml Safe-Lock tube (Eppendorf, Stevenage, UK), with 3 cycles of shaking at 20Hz for 2 minutes. Lysates were then processed as per the manufacturer's instructions, including the optional on-column DNase digestion step. The RNA was eluted in 2 x 50 µl of nuclease-free water. Further DNase digestion of the RNA solution was carried out using RQ1 RNase-Free DNase (Promega, Southampton, UK) as per the manufacturer's instructions with the sample incubated for 30 min at room temperature. To remove the DNase and reaction buffer from the purified RNA, it was passed through the RNeasy Mini Kit using the RNA clean-up protocol and was eluted in 2 x 40 µl of elution buffer (10 mM Tris HCl, pH 8.4). The RNA concentration in the eluate was measured using the Qubit RNA Assay Kit (Invitrogen, Paisley, Scotland).

Primers and probes (Table 7.2) were designed using Primer 3 (Rozen & Skaletsky, 2000) (http://frodo.wi.mit.edu/) and M-Fold using the bovine-specific GenBank sequences for the potential housekeeper genes: beta-2-microglobulin (*B2M*: NM 173893), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, NM 001034034), hypoxanthine phosphoribosyl-transferase 1 (*HPRT1*: NM 001034035), ribosomal protein S8 (*RPS8*: NM 001025317), succinate dehydrogenase complex, subunit A (*SDHA*: NM 174178), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

polypeptide (*YWAZ*: XM 001788370), as well as; ATPase, copper transporting, beta polypeptide (*Atp7b*: XM 002691794), copper chaperone for superoxide dismutase (*CCS*: NM 001046187), cytochrome c oxidase assembly homolog 17 (*Cox17*: XM 002684734), copper transport 1 homolog (*Ctr1*: NM 001100381), diamine oxidase (*DAO*: NM 001034361), glutathione peroxidase 1 (*Gpx1*: NM 174076), lysyl oxidase (*LOX*: NM 173932), metallothionein-1A (*Mt1a*: NM 001040492), and superoxide dismutase 1 (*Sod1*: NM 174615, as described previously (Peters *et al.*, 2004). Primer specificity was tested using the Primer BLAST algorithm (www.ncbi.nlm.nih.giv).

Table 7.2. Real-time RT-PCR primers of Cu regulatory proteins examined in aorta, duodenum, and liver of zebu (*B. indicus*) and crossbreed (*B. indicus* × *B. taurus*) heifers on a Cu-deficient diet.

Gene	Assay	Forward Primer	Reverse Primer	PS (bp)	E	PMT
Atp7b	Control	TAGAAGGCAAGATCGGGAAA	CTGGGGAGACGAGAGAAGG	435		
	qPCR	TAGAAGGCAAGATCGGGAAA	ATGTGGTCCCTGAGGTCTTG	121	94.9	87.0
B2M	Control	GTTCCATCCACCCCAGATT	TTACAGGTCTCGATCCCACTT	211		
	qPCR	GTTCACTCCCAACAGCAAGG	ACTATCCGGGGTTGTTCCA	72	98.7	82.0
CCS	Control	CTGTGGGGACCACTTTAACC	AGGCCATCACAGGAGCAG	388		
	qPCR	CAGGATCACAGGAAACTCAGG	GCTTGGGGTTCTGGAAGAG	80	96.1	85.5
Cox17	Control	TGAGTCGCAGGAGAAGAAGC	TCAGCAAGGAACTCCCAAAG	233		
	qPCR	GAAAGGAGAAGAGCAATGTGGA	ATTCACTCCCAGAGCAGACC	102	99.1	83.0
Ctr1	Control	TGGGGATGAACATGGATATG	AATGGCAATGCTCTGTGATG	549		
	qPCR	CCCAACCACTTCATCTGACC	AAAGCTCCAGCCATTTCTCC	138	99.3	83.0
DAO	Control	GCACTGTACGGAGGACACAC	CCGTTGGGGTAGAAGATGAA	350		
	qPCR	ACGCCCTCCACTACTACGAC	GGGCATCTCGAAGAGACAGA	71	96.7	86.0
GAPDH	Control	ACCAGGGCTGCTTTTAATTCT	GGTCATAAGTCCCTCCACGA	474		
	qPCR	GGGTCATCATCTCTGCACCT	GGAGGCATTGCTGACAATCT	101	99.6	84.5
Gpx1	Control	AACGTAGCATCGCTCTGAGG	AGCATAAAGTTGGGCTCGAA	203		
	qPCR	CGGGACTACACCCAGATGA	TCCTCGTTCTTGGCGTTT	113	93.5	89.5
HPRT1	Control	CCAGTCAACAGGCGACATAA	GGCTCGTAGTGCAAATGAAG	527		
	qPCR	GCGAAACTGGAAAAGCAAAA	GCCACAGAACAAGAACATTG	116	93.0	82.0
LOX	Control	ATACGGCACCGGCTACTTC	CCCTCAGCCACTCTCCTCT	348		
	qPCR	CCCCAGAGAGTGAAAAACCA	TGGCATCAAGCAGGTCATAG	139	95.7	84.0
Mt1a	Control	CTGCTTTGCCACTTGTTCTG	GCACCAGGTCAGATTGTATG	342		
	qPCR	CTGATGTCGGGGAGAACCT	AAGGTAATGTAGCACCAGGT	101	98.2	79.0
RPS8	Control	CATCTCTCGGGACAACTGG	TTCGCGTTCTTTTTCCTCTC	464		
	qPCR	CATCTCTCGGGACAACTGG	GCGTCCCAGCTCATACTTTC	88	96.7	86.0
SDHA	Control	TGCAGACCCGGAGATAAAGT	CGTACTCGTCAACCCTCTCC	391		
	qPCR	TAAACCAAATGCTGGGGAAG	CTGCATGTTGAGTCGCAGTT	97	95.1	81.5
SOD1	Control	GCAAGGCACCATCCACTT	CACCTCTGCCCAAGTCATCT	341		
	qPCR	GGATTCCACGTCCATCAGTT	GGTCTCCAACATGCCTCTCT	121	98.4	84.0
YWAZ	Control	GCTTCACAAGCAGAGAGCAA	CCGATGTCCACAATGTCAAG	367		
	qPCR	ACTGGGTCTGGCCCTTAACT	TGGCTTCATCAAATGCTGTC	98	97.9	82.0

PS= product size, E = efficiency, PMT = product melting temperature

Synthesis of cDNA was carried out with 500 ng of random hexamers using the ImProm-II Reverse Transcription System (Promega Corporation) using 600-1000 ng (liver and intestine) and 200 ng (aorta) of total RNA in a final volume of 40 µl. All reactions were prepared according to the manufacturer's instructions giving a final magnesium chloride concentration of 3 mM.

Reverse transcription was performed in a PTC-200 DNA engine (Bio-Rad Laboratories). Duplicate RT reactions were performed for each RNA sample. All cDNAs were diluted to a final volume of 200 µl (1:5 dilution) using EB Buffer (10mM Tris-HCl pH-8.4, Qiagen Ltd.) and then stored at -20 °C for future use. No template controls were performed by addition of nuclease free water in place of RNA.

Quantitative PCR (qPCR) was performed using GoTaq Colourless Master Mix (Promega). Gene specific amplification was performed using 0.2 µM of each primer, SYBR Green 1 (1:100,000 final concentration, Invitrogen), ROX (1:5000, Invitrogen) and 5 µl of diluted cDNA in a final volume of 25 µl. Magnesium chloride concentrations were adjusted to 4.5 mM in the final reaction by addition of 50 mM $MgCl_2$.

Sample incubations were performed in an MxPro 3005P (Agilent, Wokingham,Berkshire, UK) at 95 °C for 2 min and then 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec during which the fluorescence data were collected. Threshold values (Ct) for the samples were calculated using the MxPro qPCR software (ver 4.1) using the multiple experiment analyser with run-to-run variations in Ct normalised using a positive control of known copy number and ROX as a passive reference dye.

The absence of genomic contamination of the RNA samples was confirmed before to the RT reactions and none of the samples showed evidence of amplifiable genomic DNA with the SDHA qPCR assay. One qPCR reaction was run for each RT repeat resulting in 2 Ct values for each RNA sample.

To determine the most appropriate housekeeper genes for the study, all seven potential genes were quantified in six cDNA samples from each tissue type. A mean Ct value was calculated for each sample using the two measured Ct values for each sample for each of the potential housekeeper genes. The mean Ct value was converted to a relative copy

number value using the $E^{\Delta Ct}$ method (E: reaction efficiency as determined from a standard curve) with ΔCt values calculated relative to the sample with the largest Ct (fewest gene copies). The geNorm VBA applet for Microsoft Excel was used to determine the most stable genes from the set of tested genes (Vandesompele *et al.,* 2002). The three most stable housekeeper genes for the aorta samples were *B2M, HPRT1*, and *SDHA*; for the intestine were *GAPDH, SDHA* and *YWAZ*; for the liver were *B2M, HPRT1,* and *SDHA*. The three selected housekeeper genes were then quantified in the remaining samples and these genes were then used to normalize the results for the other genes quantified.

A relative copy number was calculated for each sample using the qBase applet for Microsoft Excel (http://medgen.ugent.be/qbase/) using the 3 housekeeper genes to normalise the results, using the methods described by Vandesompele *et al.* (2002). The sample with the fewest gene copies (latest Ct value) is given a relative copy number of 1 and all other samples are given values relative to this sample. This relative copy number result was used for all comparisons involving mRNA expression.

To assess reaction efficiency, a set of primers was designed for each gene target to amplify a larger fragment, which included the portion amplified by the qPCR assay. These assays were tested against a cDNA obtained from RNA extracted from each of the tissues. Products were separated by 2% agarose gel electrophoresis, purified by NucleoSpin Extract II kit (Macherey-Nagel) and then quantified using the Qubit dsDNA BR Assay (Invitrogen). The number of copies per µl of purified product was calculated and then a 1:10 dilution series from 10^7 to 1 copy per qPCR was analysed in triplicate using the qPCR assay and the reaction efficiency calculated using the MxPro software.

7.2.5. Statistical analyses

All statistical procedures were performed using the mixed model (SAS Inst. Inc., Cary, NC). For weight, BCS and plasma mineral concentrations, the model included sampling week as a continuous fixed effect, type as categorical fixed effect, and their interaction. Animal was considered as the experimental unit and was inserted as a random effect. For tissue mineral concentrations, the model included tissue and type as a categorical fixed effect and their interaction. Again, animal was incorporated as a random effect. To

compare mRNA expression and performance data, a fixed effects model was used with type included as categorical fixed effect. Associations between trace element storage and mRNA expression was evaluated using Spearman correlation tests.

7.3 Results

7.3.1. Plasma mineral concentrations

Plasma Cu concentrations evolved differently over time in the two breed types (type × week interaction; $p < 0.001$) (Figure 7.2), with concentrations in crossbred cattle decreasing more dramatically than in zebu cattle. Overall, plasma Cu concentrations decreased throughout the trial (week; $p < 0.001$). Plasma concentrations of both Fe and Mo were not affected by breed type (type × week interaction; $p = 0.8$, $p = 0.3$, respectively). Over time, plasma Mo increased (week; $p = 0.038$), whereas plasma Fe concentrations decreased (week; $p < 0.001$).

Plasma Zn concentrations changed differently over time between the two breed types (type × week interaction; $p = 0.035$). However, unlike in the case of Cu, plasma Zn concentrations seemed to differ between types at the start of the trial with a diminishing difference throughout the trial. Overall, plasma Zn concentrations were also affected by a type effect (type; $p = 0.001$), with lower concentrations in crossbreeds than in zebu heifers throughout the trial. Over time, plasma Zn concentrations decreased (week; $p < 0.001$).

Plasma Mn concentrations were not affected by breed type (type × week interaction; $p = 0.3$), but tended to decrease slightly over time (week; $p = 0.053$). Throughout the trial, plasma Se concentrations increased (week; $p < 0.001$) but did not differ between breed types (type × week interaction; $p = 0.2$). Over time, plasma Co concentrations evolved differently in both breed types (type × week interaction; $p = 0.001$), where the difference between types seemed large at the onset of the trial, but afterwards a smaller difference remained constant over time. Plasma Co concentrations were also subjected to an

overall breed effect throughout the trial ($p < 0.001$). Again, concentrations in crossbreeds were lower than in zebu heifers. Throughout the trial, plasma Co concentrations decreased ($p < 0.001$).

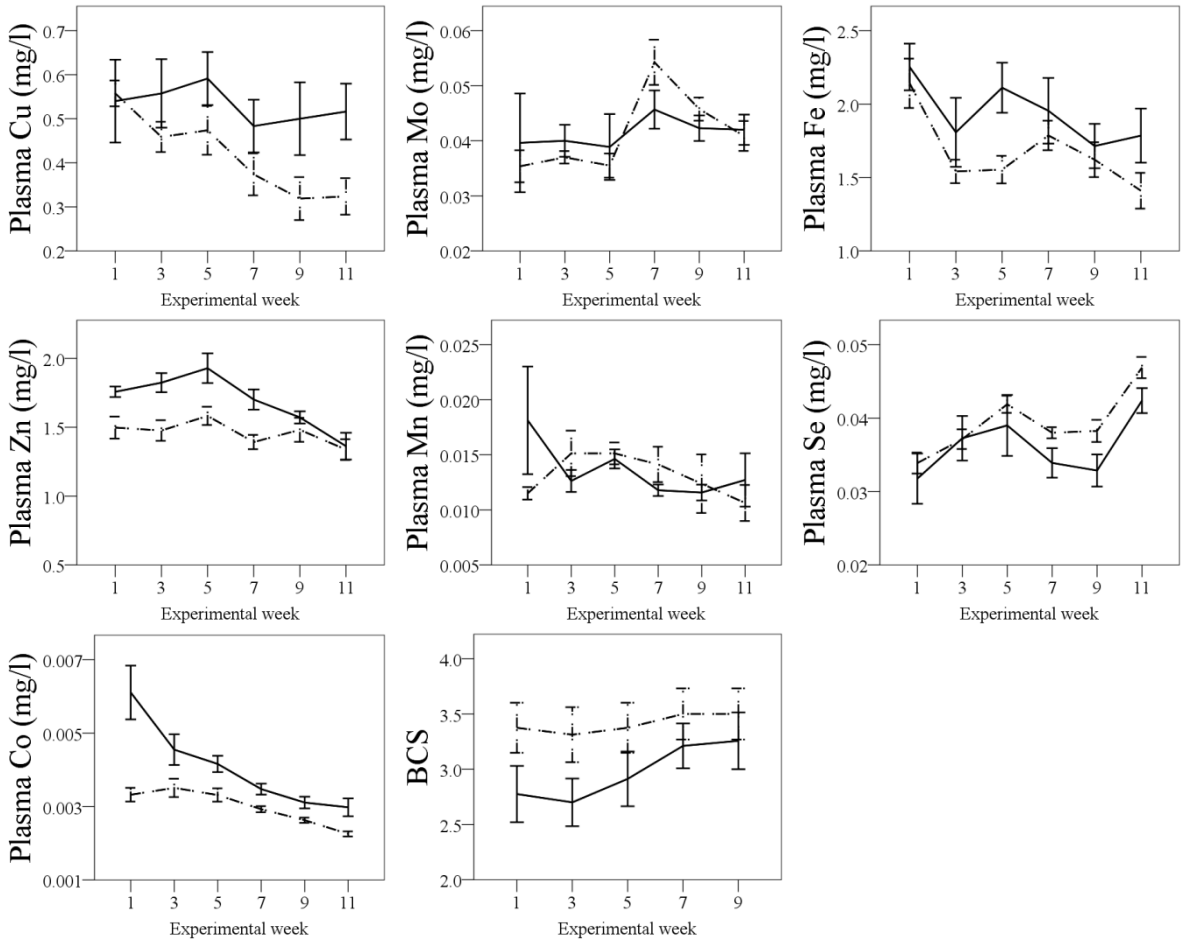

Figure 7.2. Plasma mineral concentrations and BCS in zebu (*Bos indicus*) (n = 8) and crossbreed (*Bos indicus* × *Bos taurus*) (n = 8) heifers on a Cu-deficient diet. Full lines represent zebu (*Bos indicus*) heifers, dotted lines represent crosses (*Bos indicus* × *Bos taurus*) heifers. Error bars represent ± SE. Significant time effect for all ($p < 0.050$), trend for Mn ($p = 0.050$), significant type effect for Zn and Co ($p < 0.050$), significant type × week effect for Zn, Co, Cu and BCS ($p < 0.050$).

7.3.2. Tissue mineral concentrations

The relative Cu tissue concentrations in the different organs tended to be different in the two breed types (type × organ interaction; $p = 0.055$) (Figure 7.3). Liver Cu concentrations were greater in zebu than in crossbreed heifers ($p = 0.002$). No differences were found for other tissues (cardiac muscle: $p = 1.0$, kidney and semitendinsosus muscle: $p = 0.9$).

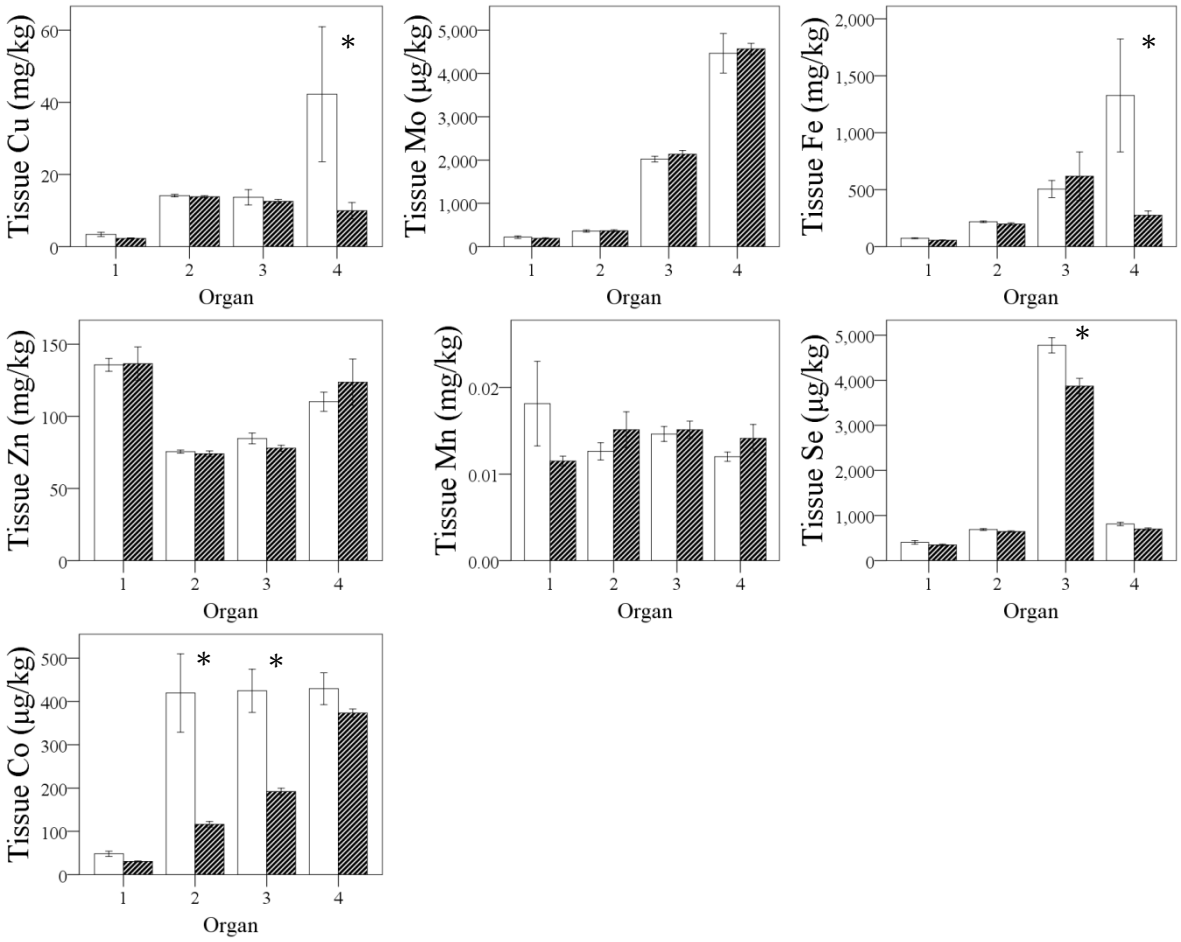

Figure 7.3. Tissue (1 = semitendinosus muscle, 2 = cardiac muscle, 3 = kidney, 4 = liver) trace element concentrations (dry weight) in zebu (*Bos indicus*) (n = 8) and crossbreed (*Bos indicus* × *Bos taurus*) (n = 8) heifers on a Cu-deficient diet. Clear bars represent zebu (*Bos indicus*) heifers, dark bars represent crosses (*Bos indicus* × *Bos taurus*) heifers. Error bars represent ± SE. Asterisks represent significant difference in tissue trace element concentrations between types of cattle ($p < 0.050$)

Relative tissue Mo concentrations were not impacted by breed type (type × organ interaction; $p = 1.0$). On the contrary, relative tissue concentrations of Fe were different in the two breed types (type × organ interaction; $p = 0.010$): Fe liver concentrations were greater in zebu than in crossbreed cattle ($p < 0.001$), other tissues were not impacted (cardiac muscle: $p = 0.9$, kidney: $p = 0.7$, semitendinosus muscle: $p = 1.0$). For Zn and Mn concentrations, no differences in tissue concentrations were found between the two types of cattle (type × organ interaction; Zn: $p = 0.6$; Mn: $p = 0.4$). Relative tissue Se concentrations differed in the two breed types (type × organ interaction; $p < 0.001$): zebu kidney Se concentrations were greater than crossbreed kidney Se concentrations ($p < 0.001$), but no differences were found for other tissues (cardiac and semitendinosus

muscle: p = 0.7; liver: p = 0.4). Finally, relative tissue Co concentrations were also affected by breed type (type × organ interaction; p < 0.001), both cardiac muscle and kidney Co concentrations were greater in zebu than in crossbreed heifers (p < 0.001).

7.3.3. Tissue gene expression

For Cu regulatory proteins, no significant type differences in relative expression of duodenal *Mt1a* mRNA were found (Table 7.3). However, hepatic mRNA expression of Cu regulatory proteins *Ctr1* (p = 0.025), *Cox17* (p = 0.009), *Atp7b* (p = 0.022) and *CCS* (p = 0.029) as well as *SOD1* (p = 0.001) were greater in zebu than in crossbred cattle. Relative expression mRNA of proteins related to Cu function, duodenal *DAO* and aorta *LOX* were not affected by type (both: p = 0.8). Type did affect relative mRNA expression of the Se related *Gpx1* in liver (p < 0.001).

Relative mRNA expression of *Mt1a* and *CCS* were correlated with liver Cu concentrations, whereas liver Mo, Co and Mn concentrations were related to duodenal *DAO* mRNA expression. Kidney Se concentrations were positively associated with liver mRNA expression of *Atp7b, CCS, Cox17, Ctr1, Sod1,* and *Gpx1* (all r > 0.5, all p < 0.050) (Table 7.4).

Table 7.3. Relative mRNA expression of trace element related proteins in tissues harvested from zebu (*B. indicus*) (n = 8) and crossbreed (*B. indicus* × *B. taurus*) (n = 8) heifers on a Cu-deficient diet.

Tissue	Gene	Zebu	Crossbreed	SEM	p
Cu regulatory proteins					
Duodenum	*Mt1a*	29	8	8	0.234
Liver	*Ctr1*	3.7	2.0	0.4	0.025
	CCS	5.0	2.6	0.6	0.029
	Cox17	2.5	1.5	0.2	0.009
	Sod1	3.4	1.6	2.6	0.001
	Atp7b	1.9	1.3	0.1	0.022
Cu related proteins					
Aorta	*LOX*	2.5	2.4	0.3	0.836
Duodenum	*DAO*	9.1	10.3	1.9	0.769
Se related protein					
Liver	*Gpx1*	3.0	1.5	0.2	<0.001

[a]Values are expressed relative to the three most stable housekeeper genes per tissue.

Table 7.4. Correlation coefficients between trace element storage and relative mRNA expression of trace element related proteins in tissues harvested from zebu (*B. indicus*) (n = 8) and crossbreed (*B. indicus × B. taurus*) (n = 8) heifers on a Cu-deficient diet.

	Aorta	Intestine		Liver					
	LOX	*DAO*	*Mt1a*	*Atp7b*	*CCS*	*Cox17*	*Ctr1*	*Sod1*	*Gpx1*
Liver Cu	0.12	0.38	0.60*	0.07	0.58*	0.17	-0.13	0.15	0.22
Liver Mo	-0.27	0.65**	-0.21	-0.26	-0.29	-0.39	-0.32	-0.28	-0.33
Liver Fe	-0.06	0.33	0.15	0.36	0.23	0.13	0.15	0.28	0.33
Liver Mn	0.01	0.58*	0.03	0.09	0.17	0.06	0.01	0.23	0.17
Liver Zn	-0.06	-0.26	0.02	-0.07	-0.12	-0.10	-0.06	-0.15	-0.17
Kidney Se	0.22	-0.09	-0.20	0.57*	0.64**	0.55*	0.59*	0.73**	0.84**
Liver Co	0.09	0.53*	0.43	0.25	0.26	0.29	0.27	0.39	0.28

*p < 0.050, **p < 0.010

7.3.4. Performance

Average daily gain seemed similar for both types (p = 0.3), but when expressed as percentage of initial body weight, zebu gained more than crosses (p = 0.014), although the latter displayed a greater absolute DM intake (p = 0.009), but not as percentage of body weight (p = 0.433). Concomitantly, the gain:feed ratio was greater in zebu than in crosses (p = 0.014) (Table 7.5). The BCS also evolved more positively for zebu than for crossbreeds (type × week interaction; p = 0.006) (Figure 7.2).

Table 7.5. Performance in zebu (*B. indicus*) (n = 8) and crossbreed (*B. indicus × B. taurus*) (n = 8) heifers on a Cu-deficient diet.

	Zebu	Crossbreed	SEM	p
Final body weight, kg	103	148	9.4	0.012
ADG, kg	0.16	0.12	0.02	0.323
DMI, kg/d	1.92	2.82	0.18	0.009
Gain:feed	0.08	0.04	0.009	0.012
DMI, % bodyweight	1.98	1.98	0.003	0.510
Total gain, % initial bodyweight	13	6.0	1.0	0.014

ADG = average daily gain, DMI = dry matter intake, Gain:feed = gain/feed ratio, SEM = standard error of means

7.4 Discussion

In the present study, zebu and crossbred cattle were kept on a Cu deficient diet, supplemented with Mo over 11 weeks to investigate the effect on plasma and tissue Cu and other trace elements and related mRNA expression. With the given dietary Mo and S concentrations and supplemented Mo, at the onset of the trial, the calculated absorption of Cu (A_{Cu}) of the unsupplemented diet, using the formula of Suttle (1983) for grass diets, A_{Cu} = 5.7 - 1.3 S - 2.785 $\log_e$ Mo + 0.227 (Mo × S), was 3.0%, whereas supplemented with 1 mg Mo/kg DM, A_{Cu} dropped to 1.8%, and when supplementation was raised to 2 mg Mo/kg DM, A_{Cu} finally decreased to 0.7%. These values in combination with the low dietary Cu concentrations inevitably lead to a very low Cu uptake from the diet, with rapidly depleting reserves in the body as a consequence. Dietary Cu forms complexes with sulfides and thiomolybdates (by interaction with Mo and S), thus rendering Cu unabsorbable. The thiomolybdates could also have been absorbed through the rumen wall, thereafter systemically binding Cu and causing a real thiomolybdate toxicity rather than a Cu deficiency (Gould & Kendall, 2011). However, the depletion of Cu reserves was reflected in the decreasing plasma Cu concentrations over time and low liver Cu concentrations at the end of the trial (overall mean: 26 mg Cu/kg DM). Mo concentrations in plasma, on the other hand, were slightly increased over time as seen by Ivan and Veira (1985): although most of the Mo will be excreted in the form of the thiomolybdates, some may also be absorbed as MoO_4 (Ferguson *et al.*, 1943). Concerning S, unfortunately, there is still no practical tool to evaluate the S status of cattle, as pointed out in **Chapter 3** and it was therefore not possible to investigate the evolution of this status over time.

Overall, the Cu deficiency caused by Mo and S interaction was evident. However, the response to the causative diet differed distinctly between the two types of cattle. Although plasma Cu concentrations were quite similar at the onset of the trial, over time they slowly decreased in *B. indicus* cattle to concentrations just below the threshold value for deficiency in *B. taurus* cattle (< 0.57 mg Cu/l; Suttle, 2010), whereas *B. taurus* × *B. indicus* crosses developed extremely low plasma Cu concentrations. Furthermore, liver

Cu concentrations were greater, yet also more variable, in zebu than in crossbred cattle (42 vs. 10 mg Cu/kg DM), 19 mg Cu/kg DM being the threshold for deficiency in *B. taurus* cattle (Suttle, 2010). Seemingly, crosses were more prone to Cu deficiency than zebu cattle. Miranda *et al.* (2010) suggested 4 different reasons for differences between cattle breeds in sensitivity towards Cu deficiency: differences in efficiency of absorption, in distribution among tissues, in excretion or in feed intake. Concerning the latter, in the present study, we saw a similar DM intake (% of BW) accompanied by a lower weight gain (% initial weight) in crossbred cattle. Therefore, the hypothesis of a greater feed intake does not seem to match with the results of our study.

Previous studies of breed sensitivity to Cu deficiency found differences between Angus and Simmental cattle, with much lower plasma Cu concentrations in Simmental cattle (Smart & Christensen, 1985). This was further investigated by Gooneratne *et al.* (1994), who found that Simmental cattle had a much greater biliary excretion of Cu. In the current study, we did not investigate differences in Cu excretion. Ward *et al.* (1995) showed that the Simmental breed seemed to have a lower apparent Cu absorption and Cu retention. Subsequently, Hansen *et al.* (2009) detected that Cu deficiency reduced mRNA expression of hepatic *Sod1*, whereas Fry *et al.* (2009) found decreased mRNA expression of *Cox17* and *Atp7b* and Hepburn *et al.,* 2009 increased expression of *CCS* mRNA in Cu deficient cattle. Fry *et al.* (2013) investigated differences in expression of Cu chaperones and transporters between Angus and Simmental cattle and detected a lower expression of duodenal copper transporters *Ctr1* and tendency for less *Atp7a* in Simmental cattle, suggesting a lower ability in these cattle to absorb and utilize dietary Cu. However, they did not detect differences in hepatic mRNA expression levels.

In our study, where the induced Cu deficiency was more severe than in the study of Fry *et al.* (2013), zebu cattle seemed to have greater relative expression of the hepatic Cu transporters and chaperones *CCS*, *Ctr1, Cox17, Sod1,* and *Atp7b* mRNA. We did not investigate the intestinal expression of these genes, but zebu and crossbred cattle may also differ in expression of intestinal Cu transporters and chaperones, which could point to better absorption mechanisms in zebu versus crossbred cattle, with greater liver and plasma Cu levels as a consequence. Although high variability in *Mt1a* mRNA expression did not allow discerning the potential differences between types, the positive

correlation with liver Cu concentrations does point in this direction. Further research is necessary to confirm this. The expression of *CCS* was also positively associated with liver Cu, in contradiction with earlier data from Han *et al.* (2009). At this point, we could hypothesise that the greater expression of the hepatic Cu transporters and chaperones suggest that the zebu cattle have a higher Cu uptake in hepatocyte (*Ctr1*), combined with a higher Cu circulation (*CCS*) and incorporation in ceruloplasmin (*Atp7b*) and cytochrome c oxidase (*Cox17*), used for scavenging of superoxide ions (*Sod1*) within the hepatocyte as well as higher Cu excretion from the hepatocyte (*Atp7b*) (Prohaska, 2004; Fry *et al.*, 2013).

An overload of Fe can exacerbate a Cu deficiency, through exchanges of Fe sulfides with Cu to unabsorbable Cu sulfides or through formation of an equally insoluble Fe-Cu-S complex (Gould & Kendall, 2011). In the present study, Fe concentrations in the diet were not as high as previously found values in grasses in the same region (**Chapter 1, 4, 5** & **7**), and the critical dietary Fe:Cu ratio was not reached (50-100; Suttle, 2010), so an additive effect of Fe on the Cu deficiency was not to be expected. On the contrary, the Cu deficiency seemed to have affected the Fe metabolism which was reflected in our study by decreasing, but not deficient, plasma Fe concentrations over time and high liver Fe concentrations (overall mean: 801 mg Fe/kg DM). Hansen *et al.* (2010) postulated that "Limited ceruloplasmin activity probably prevented Fe from being mobilized out of the liver, causing Fe to accumulate in the liver and limiting Fe availability for extrahepatic tissues", which may explain the decreasing plasma Fe concentrations noted in our study. The evolution of plasma Fe concentration over time did not differ between the two types of cattle. However, zebu cattle did have more variable and greater liver Fe concentrations than crossbred cattle, which were also greater than found in previous research in the area (**Chapter 2** & **6**). Although this difference seems to contradict the ceruloplasmin explanation of Hansen *et al.* (2010), dietary Cu levels were lower than in the supplemented study group of Hansen *et al.* (2010), and therefore, the range in Cu status range is much smaller in our study. Nevertheless, the extremely high liver Fe concentrations in zebu cattle were striking and further research is warranted to determine the physiological reason of this.

In the current study, zebu cattle had greater kidney Se concentrations than crosses and *Gpx1* mRNA expression was higher in zebu than in crosses, but no type × week interaction affected plasma Se concentrations. Langlands *et al.* (1980) previously found greater whole blood Se and *Gpx1*-Se activity in *B. indicus* (Afrikaander) than in *B. taurus* (Hereford-Shorthorn) cattle, with crosses having intermediate values. We know of no other studies investigating breed differences in bovine blood Se status, nor in kidney Se concentrations or *Gpx1* mRNA expression. We do know that Cu and Se metabolism are interrelated, with lower *Gpx1* activity, *Gpx1* mRNA expression and higher faecal excretion of Se in Cu deficient rats (Jenkinson *et al.*, 1982; Olin *et al.*, 1994). In this respect, the seemingly lower ability in crosses to cope with this Cu deficiency might have caused the lower mRNA expression of *Gpx1*. The positive association of Se storage and mRNA expression of Cu regulatory proteins, *Ctr1, CCS, Atp7b, Cox17, Sod1* suggests shared pathways between Cu and Se, yet to be elucidated. Overall, Se concentrations increased throughout the trial, which contradict the greater faecal excretion in Cu deficiency, but the rise, although significantly different over time, is small and may not be relevant. Finally, the differences between cattle types in plasma Co, semitendinosus and kidney Co concentrations may be irrespective of the Cu deficiency but warrant further research on differences in trace element metabolism between *B. indicus* and *B. taurus* types of cattle.

In conclusion, *B. indicus* and *B. taurus* × *B. indicus* cattle had a disparate response to a Cu deficient diet supplemented with Mo. Concentrations of Cu, both in transport and storage pools were greater in *B. indicus* cattle than in crossbred cattle. In *B. indicus* cattle, this coincided with a greater mRNA expression of Cu-regulatory proteins and Cu-related enzymes in the liver. This may suggest a more efficient use of dietary Cu in *B. indicus* and a lower proneness to Cu deficiency in comparison with *Bos taurus* × *Bos indicus* cattle. In *B. indicus* cattle, concentrations of Zn and Co in the transport pool and concentrations of Fe, Se and Co in certain storage pools as well as a higher hepatic mRNA expression of the Se related enzyme *Gpx1*, were also observed to be greater. Overall, future research is warranted to fully unravel these potential differences in trace element metabolism between *B. indicus* and *B. taurus* × *B. indicus* cattle and to investigate to which extent they may translate in disparate trace element requirements.

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General Discussion

The risk for trace element deficiency in cattle is omnipresent and tropical regions where zebu cattle dominate, are particularly prone to this. In this doctoral thesis, which had the Gilgel Gibe catchment as major study site, the trace element supply for grazing cattle and effect of plant type, environment and management were examined (**Chapter 1**) and the concomitant mineral status and associated animal factors were investigated (**Chapter 2**). Afterwards, the value of saliva as alternative sample for sulphur status estimation was evaluated (**Chapter 3**). Consequently, the effects of trace element supplementation in zebu cattle on anti-oxidant status and performance were studied (**Chapter 4**) as well as the effects of the same measure under practical farming conditions in crossbred cattle on trace element status and secretion, anti-oxidant status, immune response and production (**Chapter 5**). Then, trace element distribution in zebu cattle in edible tissues was examined in addition to the association of this distribution between all investigated samples (**Chapter 6**). Finally, the ability to cope with a diet deficient in trace elements was compared in zebu and crossbred cattle (**Chapter 7**).

By means of these studies, an attempt was made to formulate an answer on the following questions:

Evaluate the trace element *supply* for zebu (-influenced) cattle

Evaluate the trace element *status* in zebu (-influenced) cattle

Gain more insights on *factors* influencing the soil-plant-animal flow

Investigate the broad *effect* of status in beef and dairy zebu (-influenced) cattle

1. Evaluation of trace element supply in forages for zebu (-influenced) cattle in the Gilgel Gibe catchment, Ethiopia

1.1. Do tropical forages meet zebu requirements?

Trace element supply is known to be low in natural forages around the world. In and around Jimma, Ethiopia (**Chapters 1, 4, 5** & **7**), this is no different (Figure 1). However, it is not known whether a "deficient" or "imbalanced" supply according to *Bos taurus* requirements, could be defined as such for zebu (-influenced) cattle, as little or no research has been performed on this topic.

Nevertheless, almost all sampled forages failed to meet both beef and dairy *Bos taurus* requirements for Cu (NRC, 2000, 2001). Concentrations of Mo and S never reached toxic levels (NRC, 2005), but borderline critical Cu:Mo ratios occurred (Suttle, 2010). Such ratios, in combination with forage S concentrations above 2 g/kg DM (Suttle, 2010), will, in some cases, lower the Cu absorption through the ruminal formation of insoluble CuS and CuMoS complexes (Gould & Kendall, 2011), leading to even lower amounts of Cu available for the animal. Further, forage Fe:Cu ratios easily reached ranges with an antagonistic impact on Cu (Suttle, 2010), concomitantly lowering the hampered Cu absorption through the formation of insoluble FeCuS complexes (Gould & Kendall, 2011) and down-regulation of the intestinal non-specific divalent metal transporter (DMT1), able to absorb both Fe and Cu (Hansen & Spears, 2008). Furthermore, toxic levels of Fe (NRC, 2005) were common in the sampled forages.

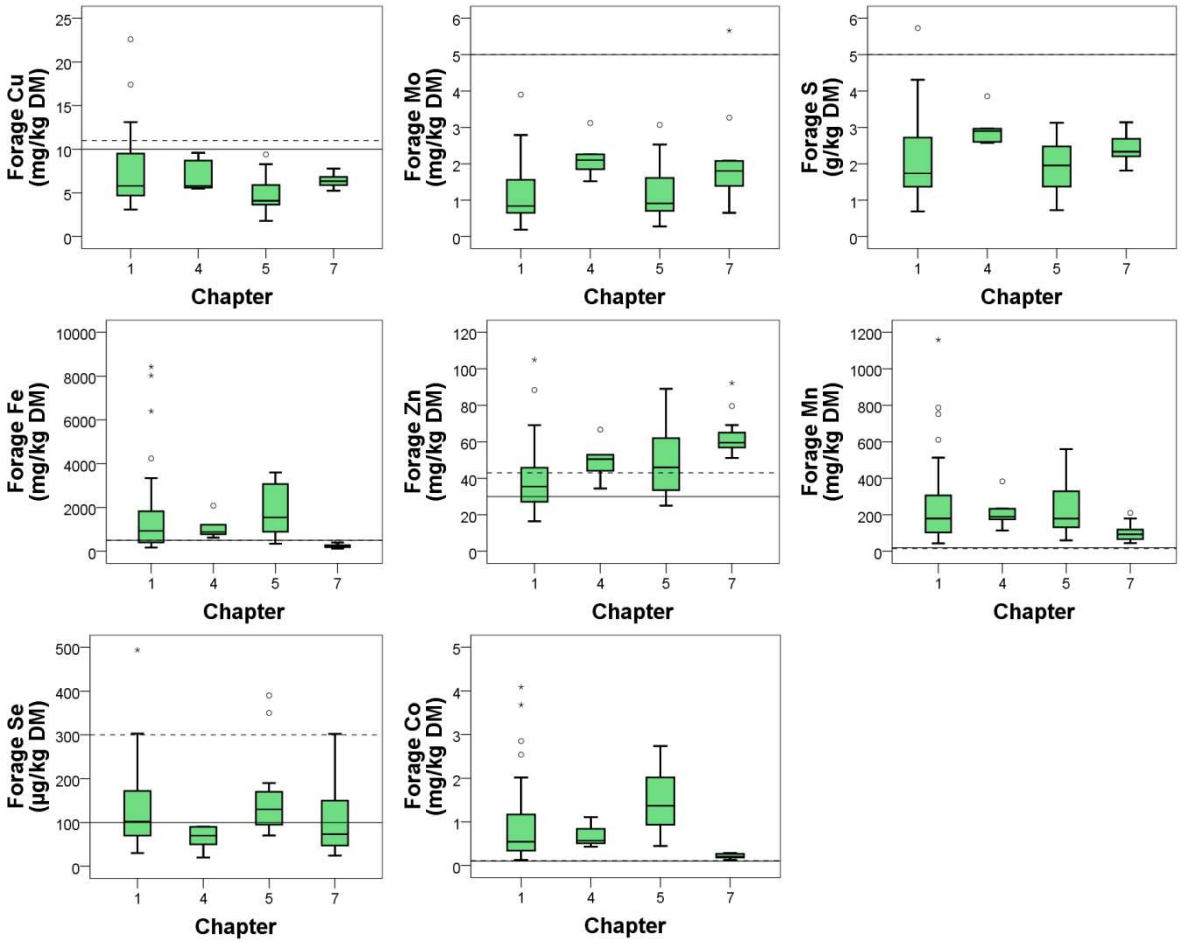

Figure 1. Box plots of trace element concentrations in forages samples (n = 69) ingested by zebu (-influenced) (*Bos indicus* and *Bos indicus* × *Bos taurus*) cattle in **Chapters 1, 4, 5** & **7**. Horizontal lines represent requirements (NRC, 2000, 2001) for Cu, Zn, Mn, Se and Co, and maximum tolerable concentrations (NRC, 2005) for Mo, S and Fe, for beef (—) and dairy (- -) (*Bos taurus*) cattle. The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values. Sampled forages represented total mixed grass diets for **Chapters 4** and **7**, whereas, in **Chapter 1** and **5**, animals also consumed other ingredients than grasses and herbaceous plants.

In general, the Se supply in tested forages was too low, especially for dairy cattle (NRC, 2001). In some cases, inadequate Zn concentrations for dairy cattle were present in forages (NRC, 2001), but generally sampled forages met *Bos taurus* requirements. Other trace elements, such as Mn and Co seemed to be adequately supplied in forages for dairy and beef cattle (NRC, 2000, 2001).

1.2. Factors related with plant trace element supply

The flow of trace elements from soil to animal is influenced by a number of factors. In the first stage of the trace element transfer from soil to animal, the soil type, closely related with elevation, appeared to be a major factor determining the estimated dietary trace element concentrations (**Chapter 1**). The trace element profile of a soil is known to be determined by certain parent layers. For instance, in the Rift Valley of East Africa, sediments of volcanic grounds with high amounts of Mo, and low concentrations of Cu, deposit and increase the risk for an imbalanced trace element supply (Faye *et al.*, 1991). Furthermore, erosion has a significant effect on plant trace element concentrations through the loss of fine soil particles which contain high amounts of trace elements (Pimentel, 2006). The humid tropics, as the studied area, are very prone to leaching and weathering of the soil (Hawando, 1997) due to certain soil characteristics, heavy rainfall and high temperatures (Conrad & McDowell, 1984). An increasing slope of the landscape is associated with higher erosion rates (Hawando, 1997) and might explain the joint impact of soil and elevation on trace element supply, seen in our data. Moreover, the presence of the Planosol-Vertisol associations, one of the two most common soil types in the area, which often flood (Van Ranst *et al.*, 2011), will have a large impact on trace element uptake by plants, with an increased uptake of Mo, a Cu antagonist. Also, this soil type has a rather neutral pH, whereas the other common soil type in the region has a more pronounced acidic pH. Again, plants growing on the first type will have a higher uptake of Mo (Tulema *et al.,* 2007). Although we did not have data on the individual impact of sub-factors, a combination of them will be highly likely to have caused the strong association between a certain soil type and plant trace element supply.

Besides the soil effect, intrinsic factors related to the sampled plants, were found to play a role in the trace element content of the sampled forages (**Chapter 1**). As in literature studying forage trace element concentrations in other areas (Abdelrahman *et al.*, 1998; Faye *et al.*, 1983; Gizachew *et al.*, 2002), trace element concentrations were highly variable in forages sampled around the Gilgel Gibe catchment. Plant types (herbaceous vs. grasses) differed largely (**Chapter 1**).

In traditional herdsmen communities of the area, herding management (e. g., grazing distance, communal grazing) and environment were also closely related, possibly indicating applied coping strategies of herders on different grazing lands and hence, different trace element supply for their cattle (**Chapter 1**), although a causal relation has not been investigated yet. If these results could be confirmed in new studies, adaptive grazing management practices could allow herdsmen to cope with poor grasslands. As such, pastoralism, often marginalized by ruling powers, might prove to make most efficient use of grazing lands in areas with environmental challenges (Catley *et al.*, 2013).

Conclusions

*The trace element supply for zebu-influenced cattle in the Gilgel Gibe catchment, Ethiopia, was imbalanced, although the applicability of Bos taurus requirements for this type of cattle was not established yet. Sampled forages contained low amounts of Cu, probably aggravated by high levels of Fe and possibly Mo and S. The trace elements Se and to a lesser degree, Zn were supplied in concentrations below requirements, especially for dairy cattle. Concentrations of other trace elements seemed sufficient for cattle (**Chapters 1, 4, 5** & **7**). Soil type, elevation and plant group seemed to be major determining factors for trace element supply. Environment and certain grazing strategies were intimately related, and might provide both explanation and solution for hampered trace element supply (**Chapter 1**). The trace element status in sampled cattle, associated with this imbalanced trace element supply is evaluated further below.*

2. Evaluation of trace element status of zebu-influenced cattle in the Gilgel Gibe catchment, Ethiopia

2.1. Liver samples

A large number (44.2 %) of liver samples of zebu (*Bos indicus*) cattle sampled around Jimma, Ethiopia (**Chapters 2** and **6**), contained concentrations of Cu which would indicate a deficient status in *Bos taurus* cattle (Puls, 1988; Suttle, 2010) (Figure 2). As explained in the Introduction, trace element status can be negatively affected by dietary antagonists. Molybdenum, in combination with S, and Fe are known to have a high antagonistic potential towards Cu (Gould & Kendall, 2011). All sampled livers had Mo concentrations below excess threshold values, whereas some but not many livers contained excess Fe concentrations (Puls, 1988).

Concentrations of Zn and Co seemed to indicate an adequate status of these elements in the sampled *Bos indicus* cattle (Puls, 1988; Suttle, 2010). For Se and Mn, as shown in the Introduction, authors disagree on hepatic threshold values/ranges for deficiency and therefore, the evaluation of the status of these elements is inconclusive (Puls, 1988; Suttle, 2010). Based upon threshold values of Suttle (2010) many cattle sampled in the region were Mn deficient but none Se deficient, although according to Puls (1988), this should be reported the other way around. We will elaborate on this subject further below.

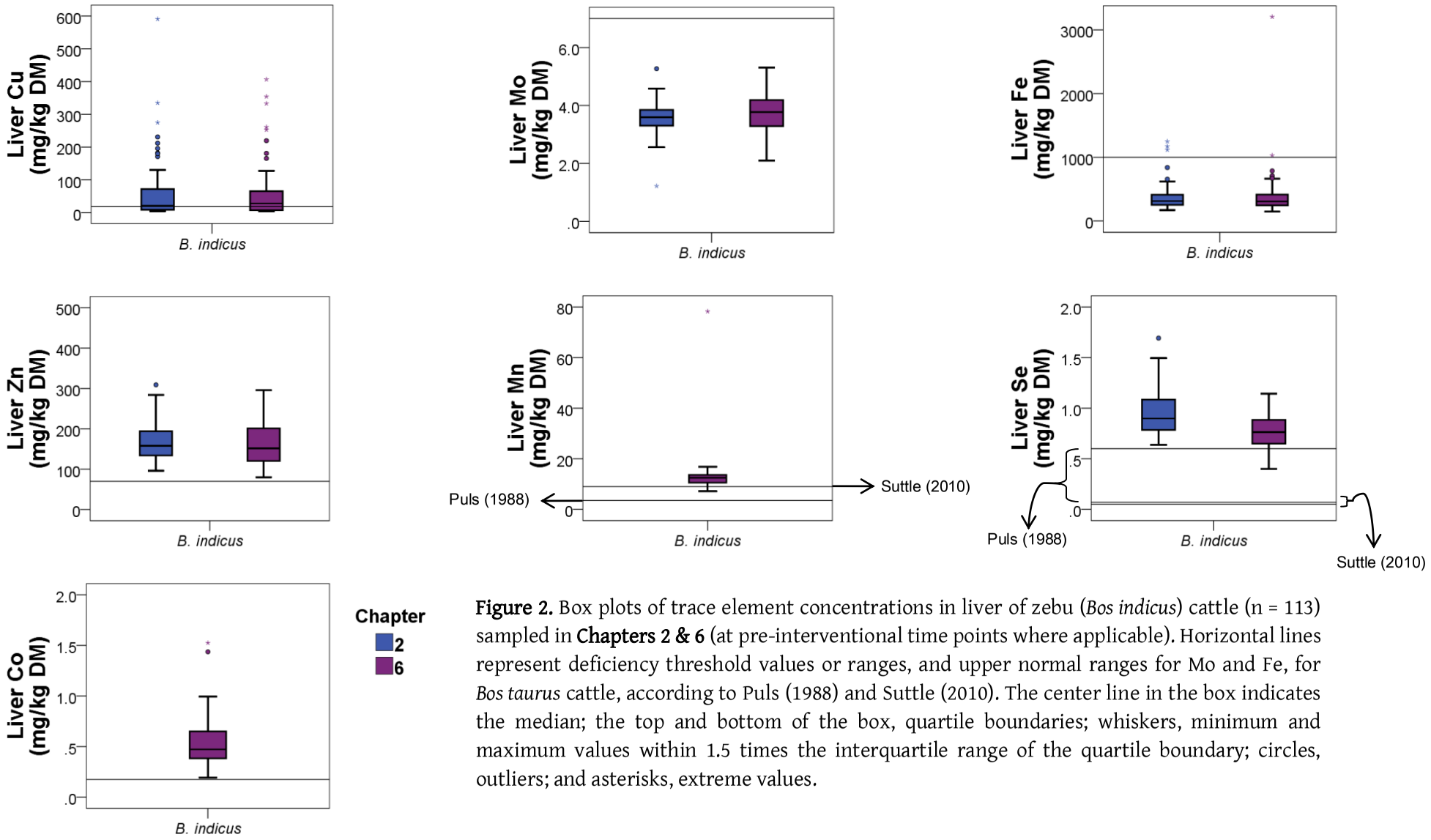

Figure 2. Box plots of trace element concentrations in liver of zebu (*Bos indicus*) cattle (n = 113) sampled in **Chapters 2 & 6** (at pre-interventional time points where applicable). Horizontal lines represent deficiency threshold values or ranges, and upper normal ranges for Mo and Fe, for *Bos taurus* cattle, according to Puls (1988) and Suttle (2010). The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values.

2.2. Plasma samples

Compiled data of plasma trace element concentrations in zebu (-influenced) cattle in and around Jimma, Ethiopia (**Chapter 2, 4, 5, 6** & **7**), largely brought the same conclusions as with liver samples (Figure 3). Many (46.8 %) cattle were Cu deficient, whereas Zn and Co status could be considered adequate based on comparison with *Bos taurus* thresholds (Puls, 1988; Suttle, 2010). As for the liver samples, for plasma samples, interpretations based on different authors resulted in conflicting reports on Mn and Se status. Based upon threshold values of Suttle (2010), at least some cattle were Mn deficient but none Se deficient, while this was reversed according to Puls (1988).

Levels of the Cu antagonists were evaluated. None of the sampled cattle had plasma Mo concentrations indicating an excessive intake, whereas all cattle exhibited elevated plasma Fe concentrations (Puls, 1988; Suttle, 2010). The status of another important antagonist of Cu, the macromineral S, remains difficult to determine. The sample of choice, rumen fluid (Suttle, 2010), is too invasive to obtain on a regular basis. Despite the well described recycling of S from blood through saliva back to the rumen (Kennedy & Siebert, 1972), we did not prove that, at higher S intakes, saliva S was a reliable indicator of S status either (**Chapter 3**). The value of plasma S was not investigated yet (**Chapter 2**). Hence, the correct evaluation of bovine S status remains challenging.

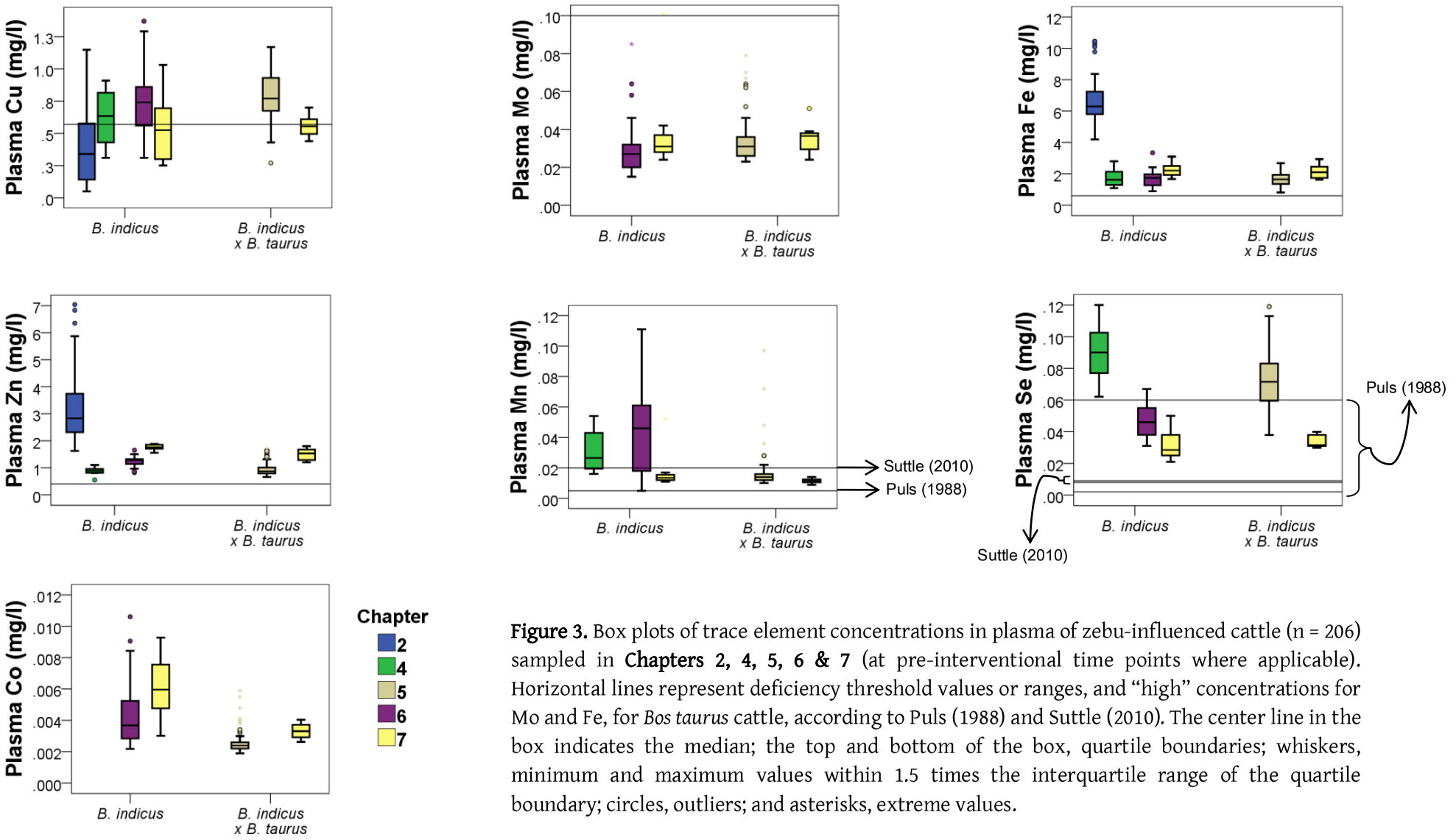

Figure 3. Box plots of trace element concentrations in plasma of zebu-influenced cattle (n = 206) sampled in **Chapters 2, 4, 5, 6 & 7** (at pre-interventional time points where applicable). Horizontal lines represent deficiency threshold values or ranges, and "high" concentrations for Mo and Fe, for *Bos taurus* cattle, according to Puls (1988) and Suttle (2010). The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values.

2.3. Association between liver and plasma samples

To assess whether plasma levels of trace elements could be used as a predictor of trace element status in zebu (-influenced) cattle, we investigated their relation with levels in liver, as main storage pool, through scatter plots and the linear regression method (**Chapters 6** & **7**) (Figure 4). Based on these scatter plots, one would suggest that only plasma Co and Se concentrations could weakly predict liver concentrations, at least when expressed by linear regression parameters. A rather curvilinear relationship was seen for Cu, in agreement with Claypool *et al.* (1975), whereas a negative association was seen for Zn, in line with Littledike *et al.* (1995).

However, as mentioned by Minatel & Carfagnini (2002), the dichotomized approach might spread a different light on the capacity of plasma concentrations to predict liver status. Using our data (**Chapters 6** & **7**), we dichotomized the outcome variable, liver trace element concentrations, as "adequate" or "deficient" (or "excess" for Mo and Fe) and calculated the ability of plasma concentrations to predict this adequate or deficient status in the liver by means of the receiver operating characteristic (ROC) method.

The high area under the curve, as seen in Figure 5, for plasma Cu concentrations, indicates that plasma Cu proves to be an excellent diagnostic test for insufficient or adequate Cu liver storage, at least for the range of concentrations found in our data. For Mn and Fe, dichotomizing the outcome variable liver did not prove to be valuable for trace element diagnostics. Other elements couldn't be tested with ROC curves due to absence of liver concentrations below or above cut-off points.

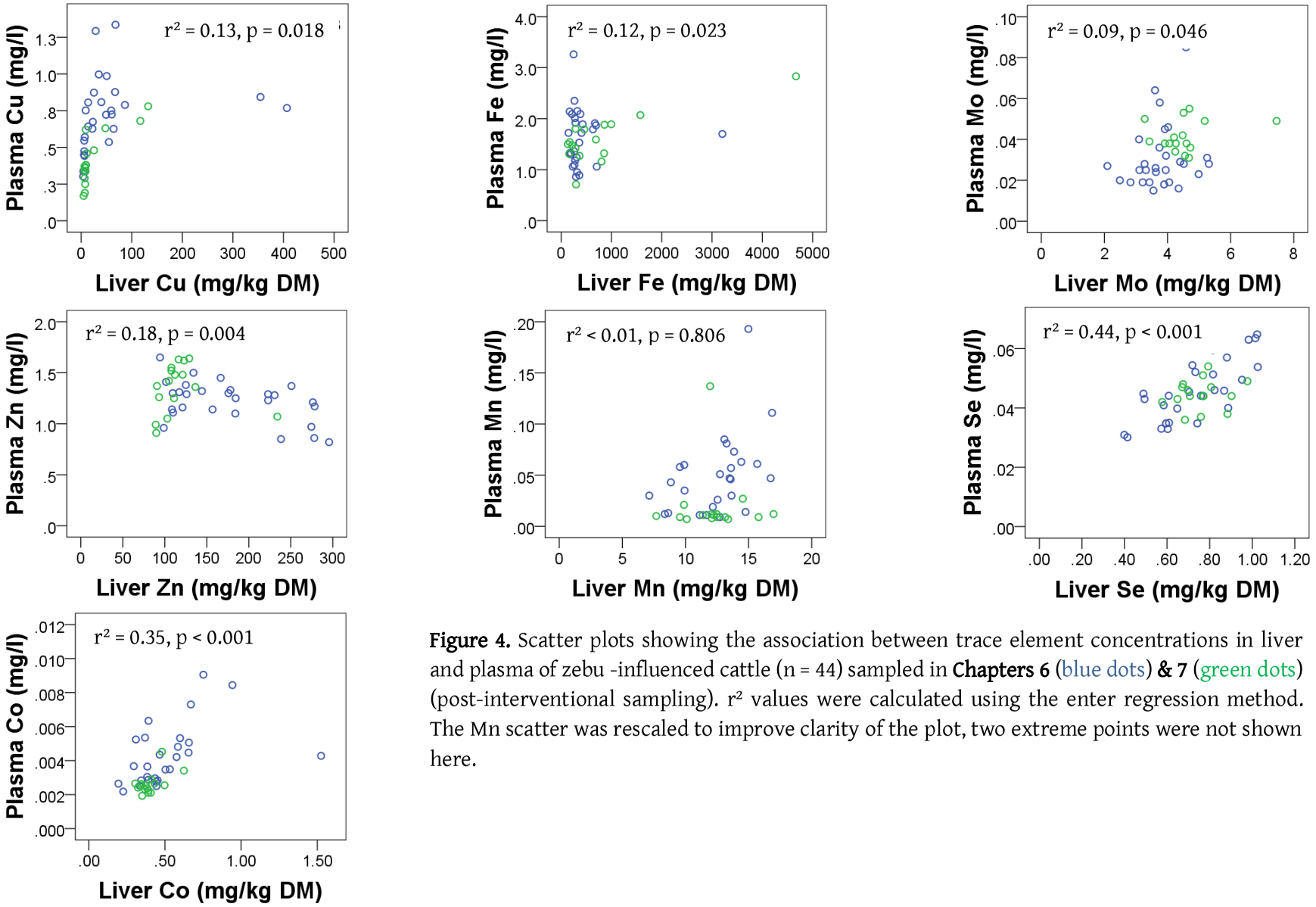

Figure 4. Scatter plots showing the association between trace element concentrations in liver and plasma of zebu -influenced cattle (n = 44) sampled in **Chapters 6** (blue dots) **& 7** (green dots) (post-interventional sampling). r^2 values were calculated using the enter regression method. The Mn scatter was rescaled to improve clarity of the plot, two extreme points were not shown here.

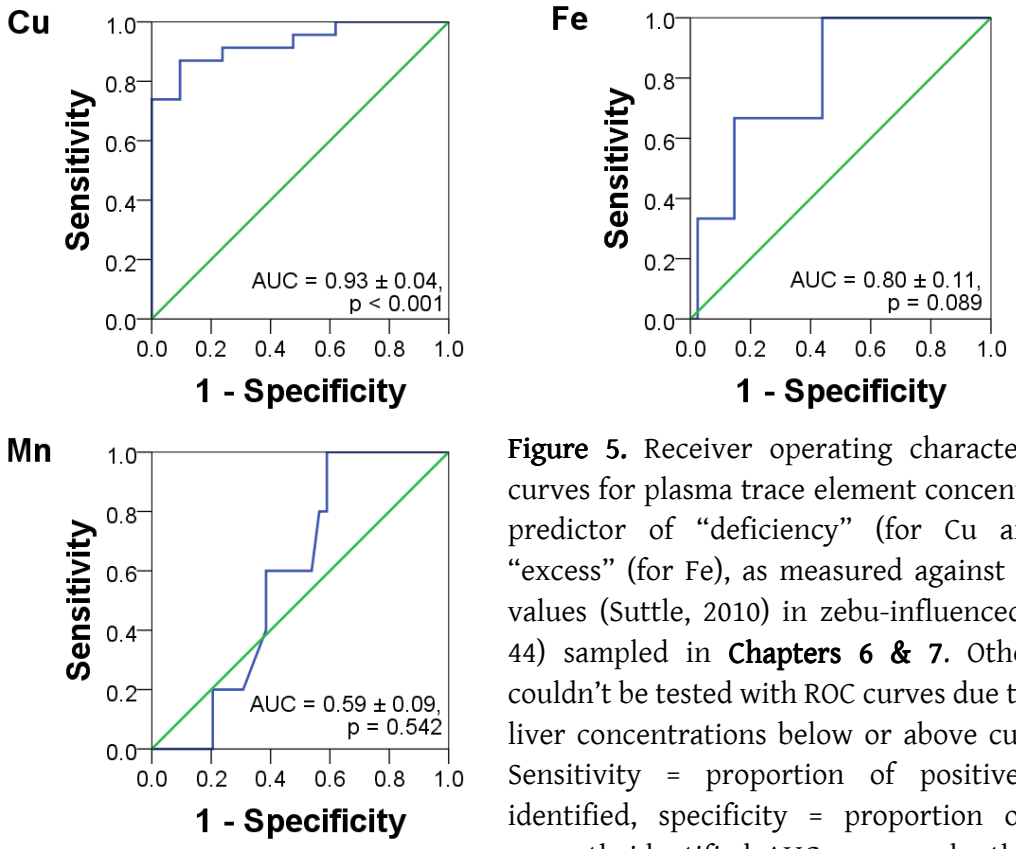

Figure 5. Receiver operating characteristic (ROC) curves for plasma trace element concentrations as a predictor of "deficiency" (for Cu and Mn) or "excess" (for Fe), as measured against liver cut-off values (Suttle, 2010) in zebu-influenced cattle (n = 44) sampled in **Chapters 6 & 7**. Other elements couldn't be tested with ROC curves due to absence of liver concentrations below or above cut-off points. Sensitivity = proportion of positives correctly identified, specificity = proportion of negatives correctly identified. AUC = area under the curve.

As seen above, the establishment of definite diagnostic thresholds for Mn and Se deficiency in cattle is problematic. At least threshold values in plasma and liver should not lead to conflicting status interpretations within authors, especially for Se, given the weak predictive value of plasma Se for liver concentrations in the linear regression model.

Judging on the higher agreement of status evaluation based on liver and plasma thresholds (**Chapters 6** & **7**), we could state that the Se status evaluation of Suttle (2010) has a better performance than the Puls (1988) evaluation (Table 1). For Mn, the Puls (1988) plasma and liver thresholds were in better agreement than plasma and liver thresholds of Suttle (2010). This, however, does not provide any information on the accuracy of these threshold values, merely on their consistency.

Table 1. Agreement of bovine Se and Mn status evaluation by means of plasma and liver thresholds stated by Suttle (2010) and Puls (1988).

	Percentage of deficiency according to author:			
	Suttle (2010)		Puls (1988)	
Trace element	Liver	Plasma	Liver	Plasma
Se	0% (0/44)	0% (0/44)	18% (8/44)	89% (39/44)
Mn	11% (5/44)	48% (21/44)	0% (0/44)	0% (0/44)

2.4. Factors related with bovine trace element status

The end stage in the flow of trace elements from soil to animal, namely an adequate bovine trace element status, is influenced by a number of factors, other than dietary supply. Soil ingestion could have exacerbated the already low Cu status because of the large amount of Fe in the soil (Suttle et al, 1984). Active geophagy was not observed in the zebu cattle sampled at the region (**Chapter 1**). However, during the dry season, dust particles were floating around and accumulated heavily on plants, with discoloration of leaves. Passive soil ingestion through this pathway might have greatly increased the intake of trace elements. In the Gilgel Gibe catchment, the reddish Nitisols, as one of two most common soil types, are renowned for high levels of Fe (Tulema *et al.*, 2007).

The influence of other factors was investigated. We compiled data from plasma trace element concentrations in sampled zebu (-influenced) cattle in the Gilgel Gibe catchment (**Chapters 4, 5, 6 & 7**). Due to unusual high plasma Fe and Zn concentrations in **Chapter 2**, as mentioned there, we excluded them from the following analyses. In the remaining pooled dataset, only weak associations were found between these plasma trace element concentrations and age. Age was positively correlated with plasma Se and Cu concentrations (Spearman correlation test, $r = 0.53$ and $r = 0.34$), whereas a negative association was found between age and plasma Zn, Co and Mo concentrations ($r = -0.34$, $r = -0.35$, $r = -0.21$ respectively) (all $p \leq 0.001$, Mo: $p = 0.035$). For Zn and Mo, this is in contrast with other data (Herdt & Hoff, 2011)

Furthermore, in the pooled dataset (**Chapters 4, 5, 6 & 7**), bulls had higher concentrations of Mn (0.064 vs. 0.017 ± 0.007 mg/l (SE)) and Co (4.5 vs. 3.1 ± 0.2 µg/l)

(ANOVA, both $p < 0.050$) than cows. In the same animals (**Chapters 4, 5, 6 & 7**), cattle were assigned a body condition score (BCS), either from 1 to 9 for zebu cattle (Nicholson & Butterworth, 1986), or from 1 to 5 for the crossbred cattle (Wildman *et al.*, 1982), scores of the latter type were recalculated to the zebu scale. Cattle with a BCS of the highest category (scores 7, 8 or 9 on the scale of 1 to 9) had the highest plasma Cu (0.84 vs. 0.70 and 0.68 ± 0.02 mg/l) and Se (0.072 vs. 0.066 and 0.053 ± 0.002 mg/l) concentrations and at the same time the lowest Zn (0.94 vs. 1.2 and 1.1 ± 0.03 mg/l) (all $p < 0.050$) and a trend for the lowest Mo concentrations (0.029 vs. 0.037 and 0.037 ± 0.001 mg/l) ($p = 0.080$) over cattle with a medium (scores 4, 5 or 6) or low BCS (scores 1, 2 or 3). Although not significantly different, we did see increasing plasma Fe concentrations as the BCS lowered (high BCS: 1.68, medium: 1.78, low: 1.87 ± 0.05 mg/l).

From most of these factors, it is not known whether these are circumstantial and confounding factors (e. g. due to higher DM intake in male animals, as suggested by Miranda *et al.* (2007) or higher requirements in female animals in reproductive stages), a consequence rather than a cause (e. f. a lower BCS because of diarrhoea in Cu deficient cattle, Mills *et al.* (1976)) or really indicating a difference related to trace element metabolism.

Besides the above mentioned factors, evidence is piling up on breed sensitivity towards trace elements deficiency. Upon comparison of plasma trace element concentrations of *Bos indicus* and *Bos indicus* × *Bos taurus* cattle sampled in the Gilgel Gibe catchment (**Chapters 4, 5, 6 & 7**), without taking any trial or diet effect into consideration, we found higher Zn (1.3 vs. 1.0 ± 0.03 mg/l), Mn (0.055 vs. 0.017 ± 0.007 mg/l) and Co (4.9 vs. 2.7 ± 0.2 µg/l) concentrations in zebu cattle (simple independent t-test, all $p \leq 0.005$), whereas higher Se (0.068 vs. 0.052 ± 0.002 mg/l) and Cu (0.76 vs. 0.68 ± 0.02 mg/l) concentrations were found in crossbred cattle ($p < 0.001$, $p < 0.050$ respectively). These differences were probably at least partly caused by differences in dietary supply, namely a more intensive feeding system with higher quality concentrates fed to the crossbred dairy cattle in **Chapter 5**, whereas zebu cattle were extensively kept, typically grazing on poor pastures. Another study, however, presented in this PhD thesis, indicated that the *B. indicus* type rather has a more efficient Cu metabolism than the *Bos indicus* × *Bos taurus* type, with higher plasma and liver trace element concentrations and a more active

mRNA expression of Cu related proteins and chaperone, when fed an identical Cu deficient diet (**Chapter 7**). In this trial, zebu cattle also had higher plasma concentrations of Zn and Co and higher tissue concentrations of Fe, Se and Co. Further research is warranted to unravel the true metabolic background of these exciting differences between cattle types.

Conclusions

*Concomitantly with the low dietary trace element supply found in the Gilgel Gibe catchment, low trace element concentrations were found in transport and storage pools of sampled zebu-influenced cattle in the region. More specifically, Cu deficiency was observed in almost half of sampled animals (**Chapter 2, 4, 5, 6 and 7**), based on status evaluation by means of plasma and liver samples. However, the value of Bos taurus threshold values necessary to assess trace element status has not been investigated yet for zebu-influenced cattle. Depending on thresholds from different authors, Mn and/or Se deficiency was present in cattle in the area. The status of other trace elements seemed adequate. Based on the tissue levels, Fe seemed the most important Cu antagonist for cattle in the region, which is in line with forage data. The status of another antagonist, S, remained difficult to determine, and saliva concentrations did not prove to be indicative of rumen fluid S concentrations (**Chapter 3**). Linear regression analysis showed that for Co and Se, plasma samples could be useful for prediction of liver values (**Chapter 6 & 7**). Using the dichotomized approach, plasma Cu concentrations proved to be very strong predictors of liver concentrations above or below cut-off point for deficiency (**Chapter 6 & 7**). Factors related with a certain trace element status (**Chapter 4, 5, 6 and 7**) were age, sex, body condition score and type of cattle. More research is needed to fully unravel the extent of the impact of these factors, particularly in zebu cattle. Moreover, the potential difference in trace element metabolism between different types (Bos indicus vs. Bos indicus × Bos taurus) of cattle (**Chapter 7**) requires extra attention, as it might have a huge impact on the optimal nutritional management of these animals.*

3. Effects of trace element status in zebu (-influenced cattle)

3.1. Performance

3.1.1. Anti-oxidant status, immunity and disease resistance

In zebu and crossbred cattle sampled around the Gilgel Gibe catchment, trace element (Zn, Cu, Mn, Se, Co and I) supplementation did not seem to affect *anti-oxidant status*. In zebu cattle, increased plasma trace element concentrations did not coincide with higher levels of superoxide dismutase (Cu, ZnSOD) or ceruloplasmine (Cp) (**Chapter 4**). For SOD, this is largely in contrast with other work supplementing Cu to cattle (Xin *et al.*, 1991; Arthington *et al.*, 1996; Ward & Spears, 1997), although Gengelbach and Spears (1998) did not observe differences in SOD either. Arthington *et al.* (1996) and (Gengelbach & Spears, 1998) saw an increase in Cp during Cu supplementation. In crossbred zebu cattle, Sharma *et al.* (2005) saw an increase in levels of both enzymes. Some authors even suggest to use Cp as an indicator of Cu reserve (Blakley & Hamilton, 1985). In comparison with that study, pooling all data from **Chapter 4** without taking any animal effect into consideration, resulted in a weak association of plasma Cu with Cp levels (Spearman correlation test, $r = 0.46$, $p = 0.001$). The cause of differences between our data and other reported results is unclear.

Furthermore, no effects of a trace element mix (Zn, Cu, Se and Co) on measures of oxidative stress (thiobarbituretic acid reactive species (TBARS)) and anti-oxidant capacity (ferric reducing ability (FRAP)) were observed in crossbred cattle kept under tropical conditions despite increased plasma trace element concentrations (**Chapter 5**). In contrast, Calamari *et al.* (2011) found lowered TBARS values in heat-stressed Se supplemented cows. Other studies investigating the effect of Cu and Zn supplementation on TBARS and FRAP studied in our work seem to be absent. However,

plasma Fe concentrations and TBARS levels were positively associated (**Chapter 5**), indicating the impact of high dietary supply of this element on oxidative stress. This is confirming earlier work, stating that, high dietary Fe levels (see section 2) are highly cytotoxic and will cause peroxidative damage to lipid membranes (Jenkins & Kramer, 1988).

Next to the lack of effects on anti-oxidant status, no effects of the trace element mix (Zn, Cu, Se, Co and I) were observed on *immunity*, measured by antibody response to rabies vaccination in crossbred dairy cattle kept in and around Jimma (**Chapter 5**), despite raised trace element concentrations in plasma of the supplemented group. This is largely in line with literature conclusions mentioned in the Introduction, stating that for Zn and Cu, no effects on humoral response are known. For Se, however, our data are contrasting the current knowledge in the field, as this element is mostly reported to affect this antibody response (Spears, 2000; Weiss & Spears, 2006; Spears & Weiss, 2008). In zebu beef cattle, Reis *et al.* (2009) did also observe a better antibody response in Se supplemented cattle than in controls and Mandal *et al.* (2007) even reported the positive effect of Zn supplementation in crossbred bulls. Other components of the immune system were considered out of the scope of this work.

Furthermore, our data also did not report any effects of the trace element mix (Zn, Cu, Se and Co) on *disease response*, as expressed by California mastitis test (CMT) scores detecting subclinical mastitis in crossbreed cattle kept under tropical conditions (**Chapter 5**), despite raised trace element concentrations in plasma in the supplemented group. Furthermore, no differences in plasma trace element concentrations were found between animals with different CMT scores at pre-interventional sampling point (ANOVA, all $p > 0.050$) (**Chapter 5**). Again, for Se, this is largely contradicting literature data (Weiss *et al.*, 1990; Spears, 2000). Positive effects of Cu and Se supplementation on somatic cell count (SCC) reduction were found in studies of Scaletti *et al.* (2003) and Weiss *et al.* (1990), respectively, whereas, Juniper *et al.* (2006) detected no differences for Se supplementation. In the study of Sobhanirad *et al.* (2010) even Zn supplementation tended to induce a CMT difference in comparison with the control group. However, under practical farming conditions, Enjalbert *et al.* (2006) did not report an increased odds ratio for mastitis in Zn or Se deficient animals and Machado *et al.* (2013) did not

find a lower odds ratio for mastitis in Se, Co, Zn and Mn treated animals. Yet, in the latter study, the treatment did influence the odds ratio for subclinical mastitis (Machado *et al.*, 2013). For more literature on this and the previous paragraph, please refer to **Chapter 5**.

It seems that there is still some gap between theory and practice, between controlled experiments and practical farming conditions, which could indicate that the full impact of management might be greater than is currently known.

3.1.2. Production

Our study investigating the short term effects of a complete trace element mix (Zn, Cu, Mn, Se, Co and I) in zebu *beef cattle*, did not indicate an effect on dietary digestibility nor on live performance parameters (**Chapter 4**) (Table 2), despite increased trace element concentrations in plasma. Mandal *et al.* (2007) observed the same in crossbred bulls supplemented with Zn. Moreover, the data obtained in this study did not show associations between pre-interventionally measured parameters and plasma trace element concentrations (Spearman correlation test, all $p > 0.050$).

Table 2. Performance parameters in control and trace element supplemented zebu (*Bos indicus*) bulls during a four weeks supplementation (Zn, Cu, Mn, Se, Co and I) trial (**Chapter 4**).

	Control	Trace element mix	SEM	p-value
Initial weight, kg	156	170	7	0.369
Final weight, kg	161	179	9	0.339
DMI, kg/d	3.2	3.5	0.2	0.419
ADG, kg/d	0.15	0.30	0.08	0.384
Gain:Feed	0.04	0.09	0.02	0.304

DMI = dry matter intake, ADG = average daily gain, SEM = standard error of means

Literature data are inconsistent on effects of trace element status on live performance characteristics in beef cattle. For Cu, some studies report positive effects (Ward & Spears, 1997; Hansen *et al.*, 2008) whereas in others, effects are absent (Ward *et al.*, 1993; Bailey *et al.*, 2001; García-Vaquero *et al.*, 2011) or even negative (Engle & Spears, 2000). In zebu cattle, Kabaija & Little (1991) did not report differences in weight gain in Cu supplemented and control bulls, but both Howard (1970) and Roeder (1980) did observe

a greater weight gain in zebu beef cows due to treatment with Cu and Co. For Zn, reported differences in live performance parameters in supplemented and non-supplemented animals, were rather small (Spears & Kegley, 2002) and both for Se and Mn, effects seem absent (Se: Droke & Loerch, 1989; Mn: Legleiter *et al.*, 2005).

In urban crossbred *dairy cattle* kept under tropical conditions, notwithstanding raised plasma trace element concentrations, a trace element mix (Zn, Cu, Se and Co) did not influence milk yield or component yields (**Chapter 5**), results comparable with other trace element supplementation studies (Cu: Engel *et al.*, 1964; Engle *et al.*, 2001; Se: Juniper *et al.*, 2006; Zn: Sobhanirad *et al.*, 2010). Also, in crossbred zebu, Zn supplementation did not induce a higher milk yield (Sharma & Joshi, 2005). However, at pre-interventional sampling a negative association existed between milk yield and plasma Mo (Spearman correlation, $r = -0.48$, $p < 0.001$). Moreover, Enjalbert *et al.* (2006) reported a significantly higher odds ratio for low milk production (as reported by the dairy farmer) in Zn deficient cattle.

More on-farm trials should aim to unravel the complex interaction between management and nutrition related to milk and component yield, resulting in either successful supplementation effects or failure to induce any effect.

Conclusions

*In zebu and crossbred cattle originating from the Gilgel Gibe catchment, Ethiopia, consuming diets low in trace elements and with an inadequate trace element status as a consequence, trace element supplementation raised trace element concentrations in the plasma transport pool (**Chapter 4 & 5**). Nonetheless, in the same cattle, we did not witness any beneficial effects of such trace element supplementation on anti-oxidant status, immunity and disease resistance or on production (**Chapter 4 & 5**). More specifically, trace element supplementation did not seem to increase levels of the antioxidant enzymes (Cp and SOD) in zebu cattle (**Chapter 4**), nor did it seem to increase measures of antioxidant power (FRAP) or decrease the amount of oxidative stress (TBARS) in crossbred cattle (**Chapter 5**). Likewise, no effect was observed on immune response (antibody titer upon vaccination) nor on disease resistance (degree of mastitis) in crossbred cattle (**Chapter 5**). Finally, trace element supplementation did not seem to affect production, neither in the more beef orientated zebu cattle (**Chapter 4**) nor in the dairy crossbred cattle (**Chapter 5**). Comparable studies in zebu cattle are largely absent. At the same time, contradicting study reports exist on almost all of the above mentioned subjects in Bos taurus cattle, probably partly because the large heterogenuity of experiments performed. Particularly, studies investigating the effects of trace element supplementation under field conditions are essential to unravel the magnitude of trace element status impact on cattle performance and production.*

3.2. Trace elements in animal products

3.2.1. Meat and organs

Reported *trace elements concentrations* in edible tissues of *B. taurus* and *B. indicus* cattle (including data from **Chapter 6**) are presented in Table 3, 4, 5 and 6.

In general, few data are available on tissue trace element concentrations, especially for *B. indicus*-cattle. Furthermore, considering differences in trace element concentrations in different types of muscles (García-Vaquero *et al.*, 2011, **Chapter 6**) and seen that authors rarely describe the specific muscle sampled, comparison of literature data for this tissue is difficult.

Table 3. Literature review of trace element concentrations in bovine liver (mg/kg WW)

Reference	Country	Cu	Mo	Fe	Zn	Mn	Se	Co
B. taurus								
Blanco-Penedo *et al.* (2006)	Spain	90	1.4	44	54	3.5	0.2	0.07
Korsrud *et al.* (1985)	Canada	28	-	-	45	-	0.3	-
López-Alonso *et al.* (2000)	Spain	60	-	-	60	-	-	-
López-Alonso *et al.*(2004)[1]	Spain	40	1.1	70	49	2.4	0.2	0.10
Nriagu *et al.* (2009)	Jamaica	20	-	-	30	-	0.4	-
Pavlata *et al.* (2001)	Czech Republic	-	-	-	-	-	0.1	-
Sedki *et al.* (2003)	Morocco	32	-	-	37	-	-	-
Waegeneers *et al.* (2009)	Belgium	80	-	-	40	-	-	-
B. indicus[2]								
Frøslie *et al.* (1983a,b)	Kenya	21	-	-	37	-	0.1	-
Khalili *et al.* (1993)	Ethiopia	4	-	293	42	4.1	-	-
Tartour (1975)	Sudan	67	-	-	-	-	-	-
Chapter 6	Ethiopia	18	1.1	118	47	3.8	0.2	0.20

[1]Geometric mean, [2]Presumably *B. indicus* cattle based upon location or mentioned as such

Table 4. Literature review of trace element concentrations in bovine kidney (mg/kg WW)

Reference	Country	Cu	Mo	Fe	Zn	Mn	Se	Co
B. taurus								
Blanco-Penedo *et al.* (2006)	Spain	4.6	0.5	59	26	1.2	1.4	0.03
Korsrud *et al.* (1985)	Canada	5.4	-	-	22	-	0.8	-
López-Alonso *et al.* (2000)	Spain	3.7	-	-	22	-	-	-
López-Alonso *et al.* (2004)[1]	Spain	3.1	0.3	51	15	0.7	1.0	0.04
Nriagu *et al.* (2009)	Jamaica	3.9	-	-	20	-	1.0	-
Sedki *et al.* (2003)	Morocco	7.3	-	-	20	-	-	-
Waegeneers *et al.* (2009)	Belgium	5.0	-	-	18	-	-	-
B. indicus × *B. taurus*								
Benemariya *et al.* (1993)[b]	Burundi	3.4	-	-	23	-	1.4	-
B. indicus								
Chapter 6	Ethiopia	3.3	0.5	97	27	1.3	1.1	0.10

[1]Geometric mean, [2]Presumably *B. indicus* crossbred cattle based upon location or mentioned as such

In sampled zebu cattle originating from different places in the Gilgel Gibe catchment, Ethiopia, low tissue levels of Se and especially Cu were found, in combination with high levels of Fe (**Chapter 6**). In literature, reported tissue concentrations of Cu

concentrations seem to be generally low or at the lower border of adequacy reported by Puls (1988), especially in *B. indicus* cattle. On the contrary, in literature, liver, kidney and muscle Fe seem to be at the higher end of or above adequacy ranges stated by the same author (Puls, 1988).

Table 5. Literature review of trace element concentrations in bovine muscle (mg/kg WW)

Source	Country	Cu	Mo	Fe	Zn	Mn	Se	Co
B. taurus								
Cabrera *et al.* (2010)[1]	Uruguay	0.4	-	42	25	0.2	0.62	-
de Freitas et al. (2014)[2]	Brazil	-	-	13	34	-	-	-
Duckett et al. (2009)[a,2]	USA	-	-	17	41	-	-	-
García-Vaquero *et al.* (2011)[1]	Spain	0.8	0.13	19	35	0.1	0.10	0.004
Huerta-Leidenz *et al.* (2003)[a,2]	Venezuela	0.8	-	19	41	0.3	-	-
Leheska et al. (2008)[a,4]	USA	0.7	-	20	41	0.1	0.18	-
López-Alonso *et al.* (2000)[3]	Spain	1.3	-	-	53	-	-	-
López-Alonso *et al.* (2004)[b,3]	Spain	1.7	0.09	39	50	0.2	ND	0.016
Pavlata *et al.* (2001)[3]	Czech Republic	-	-	-	-	-	0.04	-
Sedki *et al.* (2003)[4]	Morocco	1.0	-	-	27	-	-	-
Waegeneers *et al.* (2009)[4]	Belgium	1.6	-	-	43	-	-	-
B. indicus × *B. taurus*								
Benemariya *et al.* (1993)[c, 4]	Burundi	1.1	-	-	54	-	0.20	-
Cabrera *et al.* (2010)[1]	Uruguay	0.6	-	38	24	0.5	0.55	-
de Freitas et al. (2014)[2]	Brazil	-	-	13	35	-	-	-
Giuffrida-Mendoza *et al.* (2007)[2]	Venezuela	0.9	-	18	38	0.1	-	-
B. indicus								
Chapter 6[1]	Ethiopia	0.7	0.07	29	27	0.8	0.10	0.020

[a]Unsure whether *B. taurus* or crossbred, [b]Geometric mean, [c]Presumably *B. indicus* × *B. taurus* crossbred cattle based on location, [1]Semitendinosus muscle, [2]Longissimus dorsi thoracis muscle, [3]Diafragm muscle, [4]Not-specified

Adequate concentrations are generally observed for Zn, whereas Mn concentrations are rather low and Se often too low in comparison with ranges for adequacy of Puls (1988). This all might point to a generalized trace element imbalance in the cattle sampled in literature. However, it might also indicate that the ranges mentioned by Puls (1988) need to be re-evaluated, as they might not reflect bovine trace element status well. The

need for clarification is urgent as such reported ranges have a large impact on how we evaluate tissue concentrations in cattle.

Table 6. Literature review of trace element concentrations in bovine heart (mg/kg WW)

Reference	Country	Cu	Mo	Fe	Zn	Mn	Se	Co
B. taurus								
García-Vaquero *et al.* (2011)	Spain	4.4	0.02	45	17	0.3	0.3	0.01
B. indicus × B. taurus								
Benemariya *et al.* (1993)[1]	Burundi	4.0	-	-	20	-	0.3	-
B. indicus								
Chapter 6	Ethiopia	3.5	0.09	49	18	0.6	0.2	0.06

[1]Presumably *B. indicus × B. taurus* crossbred cattle based on location

Furthermore, few studies have investigated potential differences in trace element distribution both within the *Bos taurus* type of cattle and between *Bos taurus* and *Bos indicus* cattle. When comparing literature data on *B. taurus* and *B. indicus* cattle, liver and kidney Fe concentrations as well as cardiac Mo concentrations seemed higher in *B. indicus* than in *B. taurus* cattle. Moreover, seemingly, tissue Co concentrations are often higher in *B. indicus* than in *B. taurus* cattle (see also **Chapter 7**). Tissue concentrations in *B. indicus* and *B. indicus × B. taurus* crossbred cattle also reacted remarkably different to the prolonged consumption of a Cu deficient diet (**Chapter 7**).

In the above mentioned cattle, sampled in Ethiopia, we investigated the association between trace element concentrations in liver as the main storage organ and other tissues, in order to unravel the *impact of trace element status* on tissue distribution (**Chapter 6 & 7**). For this analysis, we excluded all values below detection limits. Overall, concentrations of trace elements in zebu (-influenced) kidney, semitendinosus muscle and heart mirrored liver concentrations very poorly (most $r^2 < 0.20$). Few exceptions were present, with Co concentrations in semitendinosus muscle and kidney being a reasonable reflection of liver Co status ($r^2 = 0.52$, $r^2 = 0.49$, respectively, both $p \leq 0.001$) and cardiac concentrations of Se representing liver Se status reasonably well ($r^2 = 0.52$, $p < 0.001$). To a much lower degree, Se in semitendinosus muscle, Co in cardiac muscle and Zn in kidney were indicative of liver Se, Co and Zn status respectively ($r^2 = 0.24$, $r^2 = 0.18$, $r^2 = 0.23$, all $p \leq 0.001$).

However, as mentioned above, linear regression analysis is not the only tool to analyse associations and using other methods might spread a different light on this subject. When using Spearman correlation tests, we generally saw a greater coherence between liver status and tissue distribution as already emphasized in **Chapter 6**. Results from the compiled dataset with removed concentrations below detection limits, were comparable to those in **Chapter 6** (Table 6.3), although now, we did also see an improved association between liver status and semitendinosus muscle concentrations of Co and Zn ($r = 0.65$, $p = 0.001$; $r = -0.29$, $p = 0.010$, respectively) as well as between liver Se status and kidney Se concentrations ($r = 0.24$, $p = 0.037$).

Thus, the renally excreted Se and Co exhibit the strongest link between liver status and distribution to other tissues, which is in agreement with the statements of Windisch & Ettfie (2008). The distribution of Zn seems odd with a negative association between liver and muscle storage in line with Blanco-Penedo *et al.* (2010), but it is known that a real storage pool for Zn is absent (Suttle, 2010), as mentioned in the Introduction. For the other trace elements, failure to observe a good agreement between linear regression and Spearman correlation results might, among others, indicate a non-linear relation between the two variables. For these elements, Cu, Fe, Mn and Mo, this non-linear association may adhere to the theoretical curvilinear pattern of distribution between liver and other storage sites (Suttle, 2010; Herdt & Hoff, 2011), as demonstrated in the Introduction. Overall, trace element status is definitely affecting tissue storage and therefore the trace element supply for human nutrition.

To evaluate the potential value of Ethiopian zebu tissue for *human trace element supply*, we compared trace element concentrations in 100 g of tissue (**Chapter 6**), called a portion, with recommended daily allowance for adult men and women as advised by the Food and Nutrition Board and Institute of Medicine (2000a,b) (Figure 6 & 7).

Overall, portions of kidney and liver could contribute a reasonable amount of trace elements for human nutrition. For instance, daily recommendations for Mo and Se would easily be met through consumption of 100 g of liver and kidney respectively. Moreover, a portion of liver and kidney could provide enough Fe for men, while

menstruating women would require additional sources of this element (Food and Nutrition Board, Institute of Medicine, 2000a,b).

In contrast, the two muscle types were generally poor suppliers of trace elements with the cardiac muscle being a slightly better supplier for almost all trace elements. A portion of zebu semitendinosus muscle generally contributed similar amounts for human nutrition as reported by Cabrera *et al.* (2010), although Se and Fe were lower in our study (**Chapter 6**). Generally, daily Zn and Mn requirements could not be reached through consumption of 100 g of any the sampled tissues (Figure 7). Equally important, Figure 7 also calls for a nuanced assessment of trace element supply for humans through zebu tissue, as for some tissue trace elements, concentrations were very variable which would lead to an inaccuracy when simply using average trace element content. For instance, on average, consumption of 100 g of liver would supply enough Cu for both men and women, although such a portion did not meet human Cu requirements in the majority of sampled livers (Figure 7).

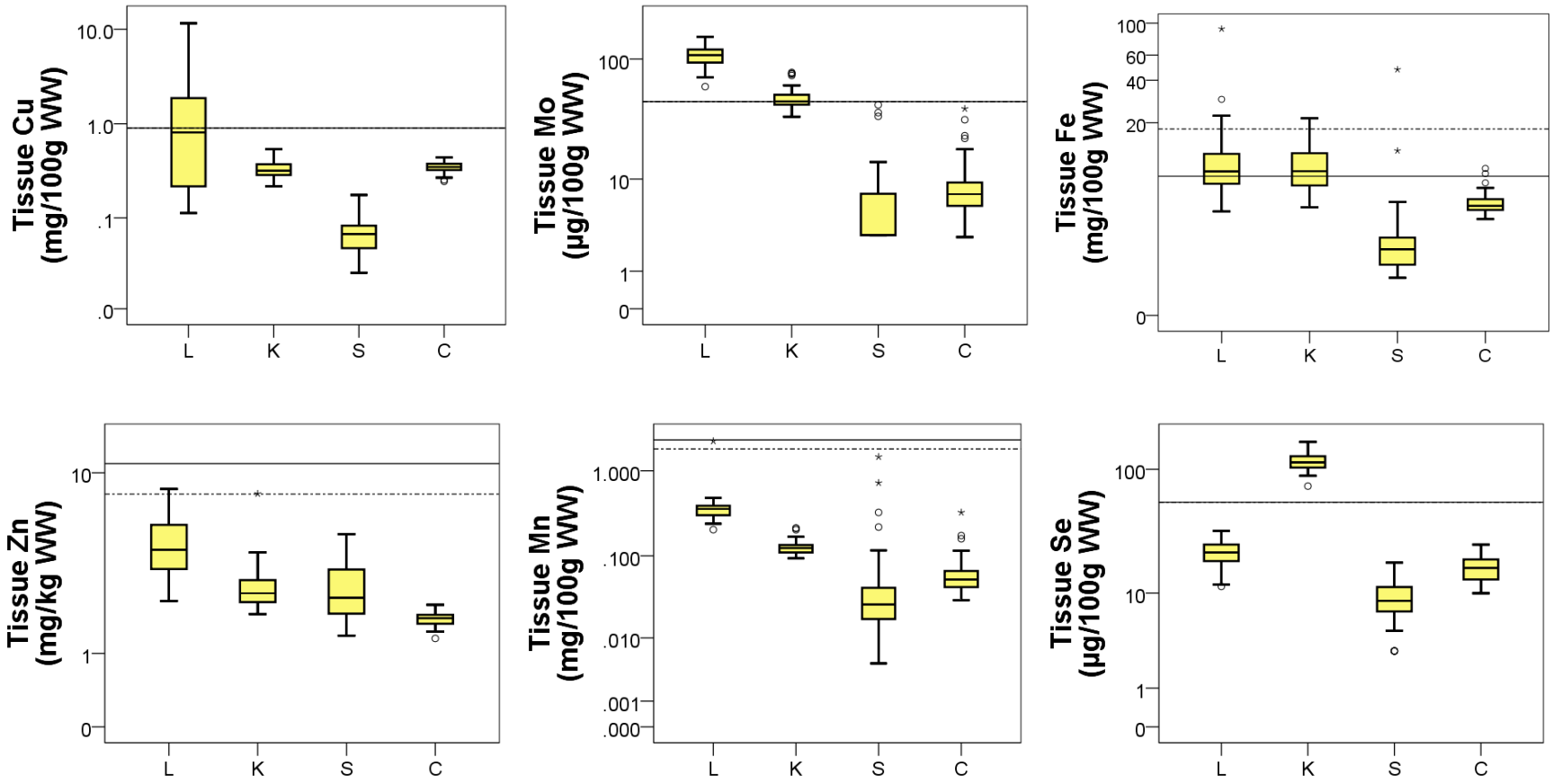

Figure 6. Box plots of trace element contents in 100g of fresh tissue matter (L = liver, K = kidney, S = semitendinosus muscle, C = cardiac muscle) sampled from zebu bulls (n = 60) originating from the Gilgel Gibe catchment, Ethiopia (**Chapter 6**). Horizontal lines represent recommended daily allowance for — = adult males and - - - = for adult females according to Food and Nutrition Board, Institute of Medicine (2000a, b). The center line in the box indicates the median; the top and bottom of the box, quartile boundaries; whiskers, minimum and maximum values within 1.5 times the interquartile range of the quartile boundary; circles, outliers; and asterisks, extreme values. Concentrations are presented on a logarithmic scale to improve clarity.

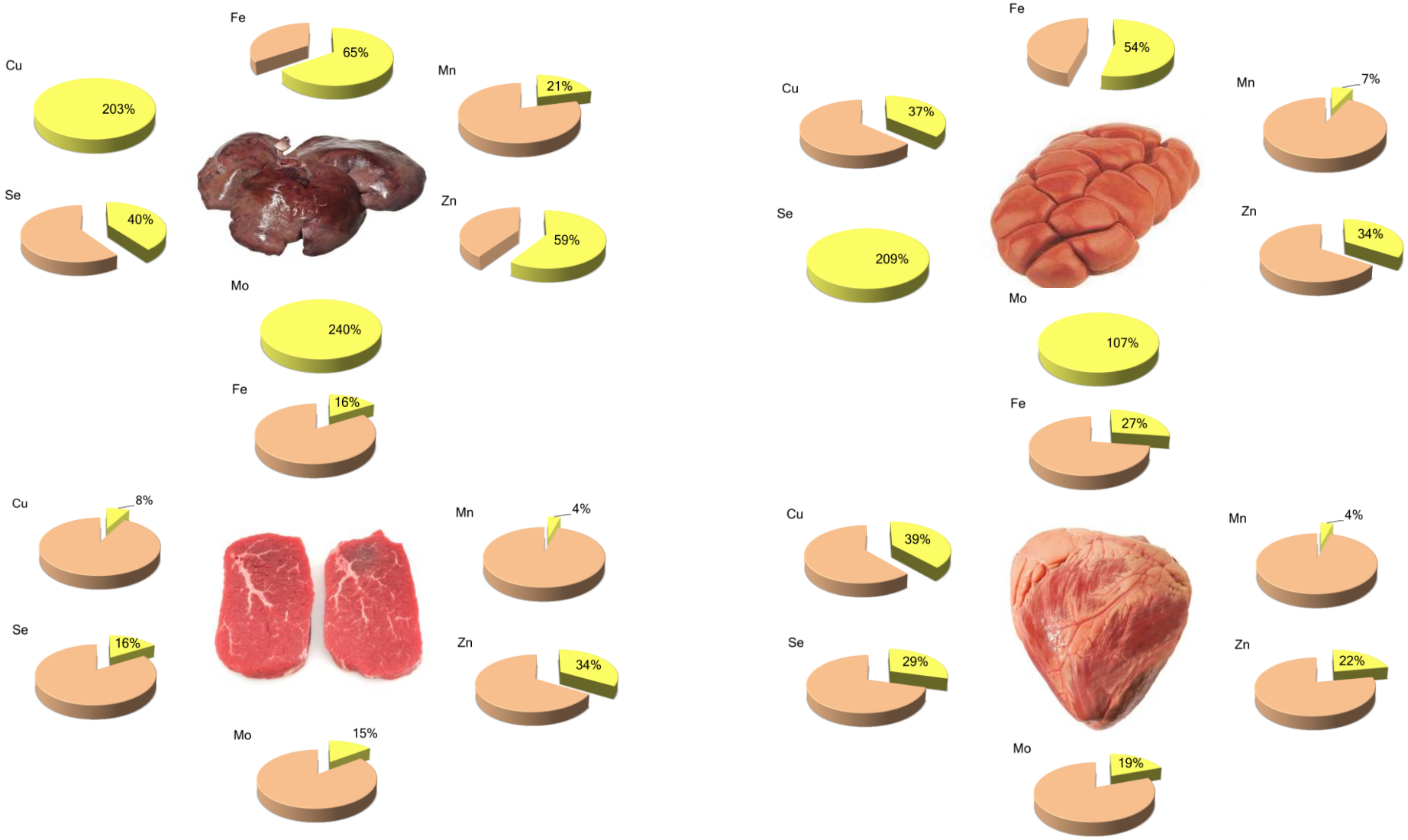

Figure 7. Average potential contribution of 100g of fresh tissue (clockwise from the upper left corner: liver, kidney, cardiac and semitendinosus muscle) matter sampled from zebu cattle originating from the Gilgel Gibe catchment, Ethiopia to recommended daily allowance of trace elements for humans (♂,♀; Zn, Mn, Fe, ♀) according to Food and Nutrition Board, Institute of Medicine (2000a,b).

3.2.2. Milk

Crossbreeding *B. indicus* and *B. taurus* is mainly executed in order to obtain a higher production and reproduction. In the case of crossbreeding zebu with Holstein Friesian cattle, a higher milk production is the main objective (Abraha *et al.*, 2009; Fekadu *et al.*, 2011). In crossbred urban dairy cattle sampled around Jimma, Ethiopia, milk samples generally contained normal *levels of trace elements* upon comparison with adequate ranges stated by Puls (1988) (**Chapter 5**), although Cu and Se concentrations were rather low and Mn concentrations rather high. However, our results are quite similar to values reported in other studies (Table 7).

Studies investigating milk mineral levels in *B. indicus* (or crossbreeds) are rare, rendering a comparison difficult. Concentrations of trace elements in milk in *B. taurus* and *B. indicus* (or crossbreeds of the latter) seem generally quite similar, but concentrations of Mn seem higher in zebu (-influenced cattle).

Table 7. Literature review of trace element concentrations in bovine milk (mg/l)

Reference	Country	Cu	Mo	Fe	Zn	Mn	Se	Co
B. taurus								
Flynn (1992)	Several	0.09	0.05	0.50	3.5	0.03	0.010	0.001
Fransson & Lönnerdal (1983)	USA	0.10	-	0.29	3.5	-	-	-
Rey-Crespo *et al.* (2013)[1]	Spain	0.07	0.04	0.35	3.9	0.03	0.019	0.005
Elmastas *et al.* (2005)	Turkey	0.07	-	-	8.2	-	-	-
Admasu *et al.* (2008)	Ethiopia	-	-	1.40	5.0	-	-	-
B. indicus × *B. taurus*								
Admasu *et al.* (2008)	Ethiopia	-	-	1.60	4.5	-	-	-
Benemariya *et al.* (1993)[2]	Burundi	0.10	-	-	4.4	-	0.026	-
Raghu (*in press*)	India	0.15	-	-	3.7	0.12	-	-
Chapter 5	Ethiopia	0.09	0.06	0.59	4.0	0.10	0.022	0.005
B. indicus								
Murray *et al.* (1980)	Kenya	-	-	0.34	-	-	-	-
Raghu (*in press*)	India	-	-	-	4.9	0.09	-	-
Salih *et al.* (1987)	USA	0.32	0.04	0.51	4.2	0.09	0.010	-

[1]Geometric mean in supermarket milk, [2]Presumably *B. indicus* × *B. taurus* crossbred cattle, based on location

In the sampled crossbred dairy cattle, we also investigated the *impact of trace element status* on milk trace element concentrations (**Chapter 5**) (Table 8). No liver samples, considered the golden standard for evaluating bovine trace element status, were

obtained in this study, hence, we employed plasma concentrations as proxy. Using linear regression, milk concentrations of almost all trace elements reflected plasma status very poorly (all $r^2 < 0.10$, $p > 0.050$). As an exception, milk concentrations of Se, mirrored plasma concentrations reasonably well, whereas for Co, only a poor relation was found ($r^2 = 0.39$, $r^2 = 0.11$ respectively, both $p < 0.001$). Results of Spearman correlation tests largely brought the same conclusions, except for milk Cu, of which concentrations were also significantly correlated with plasma Cu concentrations (Table 8). Our results are in line with earlier research for Co and Se, although the link found in our data is stronger for Se (Se: Knowles *et al.*, 1999: $r^2 = 0.24$; Co: Kincaid & Socha, 2007: no r^2 calculated).

Table 8. Spearman rank coefficients for correlation between plasma and milk mineral concentrations (**Chapter 5**)

		Plasma						
		Cu	Mo	Fe	Zn	Mn	Co	Se
Milk	Cu	0.234**						
	Mo		0.007					
	Fe			0.150				
	Zn				0.077			
	Mn					0.151		
	Co						0.192*	
	Se							0.591**

*p < 0.050, **p < 0.001

Milk of *B. indicus* × *B. taurus* cattle sampled in and around Jimma, Ethiopia, generally contributed very little to meet *human requirements* of Cu, Fe and Mn (**Chapter 5**) (Figure 8). On the contrary, milk seemed a good supplier of Zn and Se for human nutrition and very large supplier of Mo. The Cu and Fe findings are generally in agreement with the calculations of Cashman (2006) for *Bos taurus* cattle, but contributions of Zn, Mo and Mn seem much higher. Yet, for some elements, milk concentrations were variable and should again be used with care when considering the potential contribution to human nutrition.

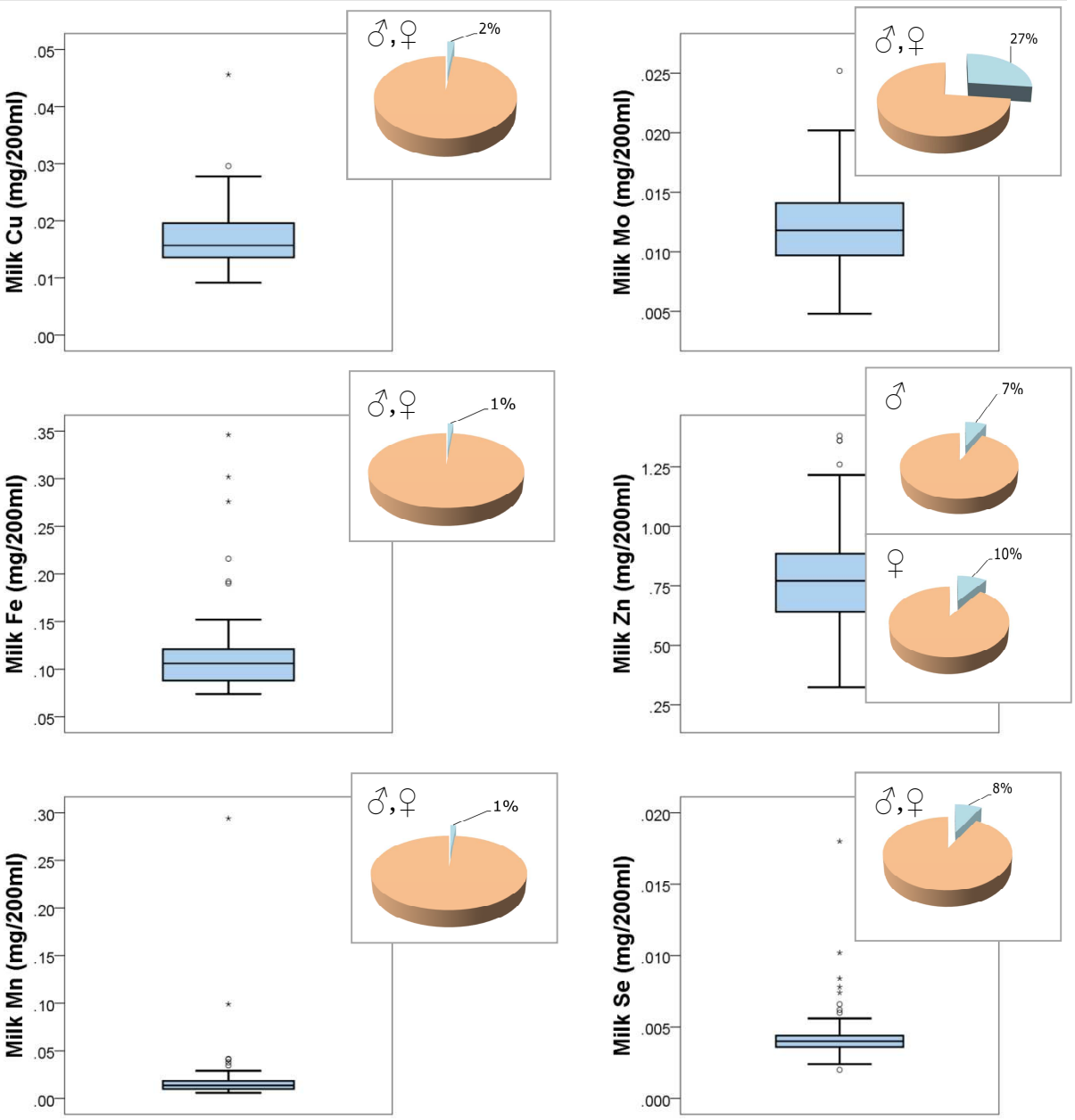

Figure 8. Trace element contents in 200ml of *B. indicus* × *B. taurus* crossbred cattle (pre-interventional time-point) (**Chapter 5**) and the contribution of the mean trace element concentrations for human nutrition, according to requirements established by Food and Nutrition Board, Institute of Medicine (2000a,b).

Conclusions

*In zebu and crossbreed cattle grazing at the Gilgel Gibe catchment, Ethiopia, low dietary supply of trace elements and low trace element status coincided with lowered trace element distribution and secretion (**Chapter 5 & 6**). In zebu cattle, low tissue levels of Cu and Se were observed, whereas Fe levels were rather high, based upon comparison with reported ranges for Bos taurus cattle and other literature (**Chapter 6**). Based on ranks, for most elements, kidney trace element concentrations were reasonably associated with trace element status. For concentrations in muscle (semitendinosus and cardiac muscle), this was less pronounced, but a strong association with status was observed for Co and Se (**Chapter 6**). On average, the potential contribution of a portion of kidney and liver to human trace element nutrition was large, whereas both muscle types were poor suppliers of trace elements for humans (**Chapter 6**). In crossbred dairy cattle, milk concentrations of Cu and Se were low and Mn concentrations high, but all were within reported normal ranges (**Chapter 5**). Based on ranks, for most elements, a poor association was observed between milk trace element concentrations and trace element status, except for milk Se, which corresponded reasonably well with status (**Chapter 5**). A glass of milk was a rather poor supplier of trace elements for human nutrition, except for Mo (**Chapter 5**). More studies are needed to unravel the factors which contribute to any remaining variation in the association between trace element status and trace element distribution and secretion.*

4. Future perspectives

As demonstrated in this PhD thesis, the low trace element supply in forages in tropical areas will inevitably cause trace element imbalances in grazing zebu cattle. Unfortunately, bovine trace element-related pathologies are neither restricted to these regions, nor to this type of cattle alone. Worldwide, both grazing and stall-fed cattle are prone to an imbalanced trace element supply, deficient or toxic, in an acute or chronic manner. Based on the compiled work, investigating trace elements in zebu cattle in a selected area with frequently observed deficiencies, we were able to shed some new light on many aspects of trace elements in cattle. Below are some proposals for continued research in this exciting field (Figure 9).

First of all, both for farmers and researchers, it is of utmost importance to be able to accurately evaluate bovine trace element status, for farmers to identify those animals suffering most from imbalanced supply and the need for intervention, and for researchers to truly investigate all effects (e.g. on health, production) of this status. Therefore, it is necessary to perform large scale studies to set or at least refine current diagnostic ranges for status, especially in liver and plasma samples, as at present, ranges for deficiency or adequacy remain ill-defined. For some elements, these samples seem poor responders to changing dietary intake. Moreover, in remote areas, preservation and analysis of these samples might be hampered. In both cases, researchers should investigate alternative samples or methods to evaluate status, adapted to practical farming.

In case of deficiency, supplementation is a valuable tool to improve trace element status, but it is often very costly to implement this measure and especially for grazing cattle, desired trace element intake is not guaranteed. In other regions, supplementation is restricted because of environmental impact concerns or a shift to organic farming systems. The present work is pointing to the intimate association between environment and management, with an impact on trace element content of the grazed diet. A different approach to trace element imbalance could be developed by scientists, taking both specific physical characteristics of grasslands and local grazing customs into account. Additionally, tracking plants accumulating trace elements and exploring their use, might offer an effective and sustainable tool to meet trace element requirements of grazing cattle, worldwide.

Furthermore, at present, a knowledge gap still exists on biochemical pathways and bodily processes affected by trace element status, as the etiology of some trace element responsive symptoms remains unclear. Also, long-term effects of suboptimal status, the stage before deficiency, are not well mapped. Consequently, fundamental research with a comparative approach across animal species continues to have its role in trace element research. Yet, the present work also suggests that despite the clear theory, with a fully unravelled mechanistic background, the practice is whimsical. Fundamental research should therefore always be tested in field trials, to investigate the nature of any loose ends between practice and theory.

Moreover, trace element status is affecting trace element concentrations in organs, meat and milk, which should be further confirmed in new studies. In spite of this work, few studies were performed to unravel complete distribution patterns during different trace element supply stages. Considering the potential contribution of these tissues for human trace element supply, surely, we should not leave such an important field uninvestigated.

Finally, we return to our starting point, both the tool and the subject of this work: trace elements in zebu cattle. At present, studies are too few to make general statements on specific characteristics of trace element metabolism in zebu cattle, but the present work does suggest that these cattle might cope differently with deficiency. In fact, to be able to fully investigate this subject, renewed knowledge on all above mentioned themes is urgently needed.

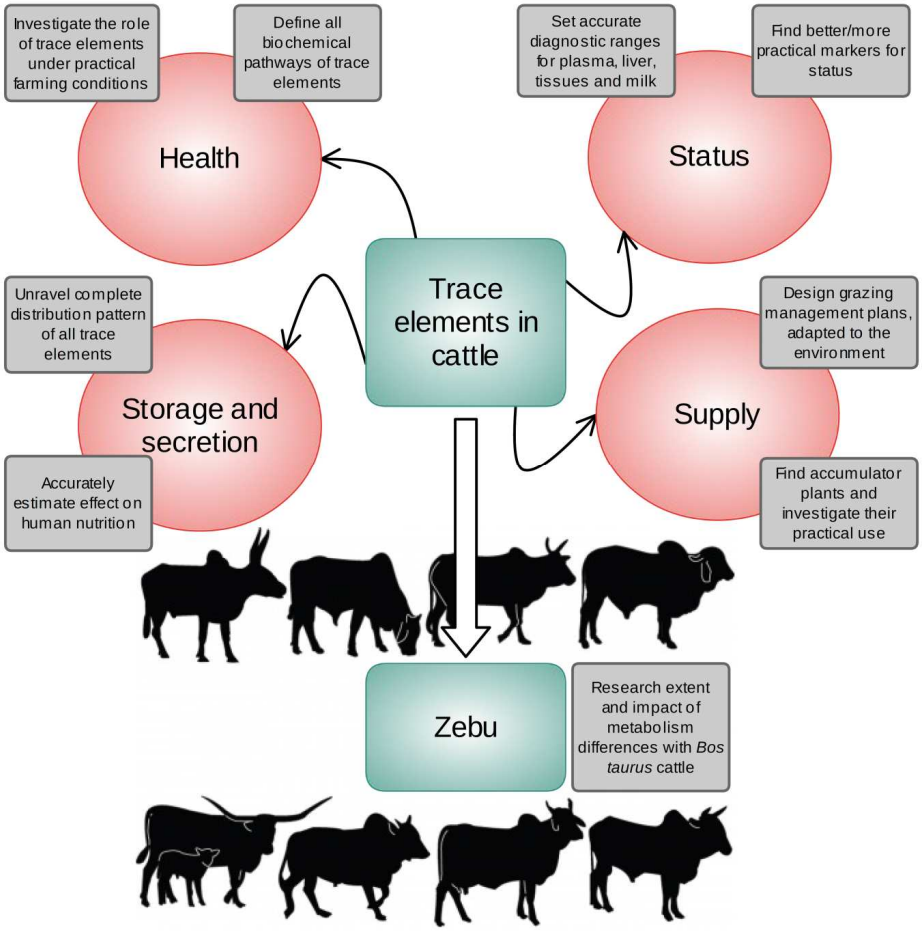

Figure 9. Future perspectives on trace element research in (zebu) cattle.

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Summary

Worldwide, grazing cattle are prone to trace element deficiencies. The **Introduction** elaborated on current knowledge on trace elements in cattle. In an ideal world, trace elements, gifts of nature, freely cycle from soil through plant to animal and back. However, such equilibrium is rarely observed. In spite of earlier research, all factors affecting the trace element flow, hampering or facilitating the achievement of an optimal bovine trace element status were not completely mapped. Furthermore, more insights are needed on the broad impact of bovine trace element status as for instance, reported effects of trace element status on immunity and production are conflicting, and rarely, these effects were studied under practical farming conditions. Additionally, the influence of trace element status on trace element concentrations in edible tissues and milk, potentially affecting human trace element supply, is not well understood. Moreover, little research has been performed on trace element nutrition in zebu cattle, the dominant cattle type in tropical regions, where a higher risk for trace element imbalance exists. Therefore, these aspects formed the main focus points of the present thesis. To fully investigate the formulated research aims, we selected the Gilgel Gibe catchment in South-West Ethiopia, as our study site.

Chapter 1 investigated the trace element content of consumed diets of grazing bovines and the effect of plant factors, environment and management on this content. Plants ingested by grazing herds of zebu cattle (n = 19) were sampled and total dietary intake was estimated through ten minute interval bite observation. In these herds, environment and management were interrelated, with the typical occurrence of communal grazing for longer distances on Planosol-Vertisol (PV) associations, at lower altitude. Many sampled plants contained inadequate Cu (72 %), Se (59 %) and Zn (43 %) concentrations, and composed diets also frequently contained disadvantageous Cu:Mo (19%) and Fe:Cu (41 %) ratios. Overall, herbaceous and woody plants contained higher amounts of trace elements than grasses and crop resides (e.g. Cu and its main antagonist, S), whereas for other elements, plant concentrations differed according to the sampled grazing altitude (e. g. Mn and Zn). Furthermore, the soil affected plant trace element supply, with plants growing on Nitisol-Acrisol-Ferralsol associations containing more Fe than those on PV associations and herds grazing on PV soils ingesting more Cu and Fe and less Mo those on NAFs. Communal grazing herds ingested

more Mo and Mn than individual grazing ones and herding distance had a positive effect on dietary Mn, whereas supplementing cattle diets with crop residues negatively affected dietary Zn and Se. Overall, environment and certain grazing strategies were intimately related, and might provide both explanation and solution for hampered trace element supply.

Consequently, **Chapter 2** aimed to evaluate the mineral status of grazing zebu cattle and to unravel associated factors. In two studies, individual plasma and pooled herd faeces were sampled in adult zebu cattle grazing at the catchment (n = 90) and liver in adult zebu bulls slaugthered at the local abattoir (n = 53). Plasma and liver analyses revealed a Cu deficiency problem in the area, since 68 % and 47 % of cattle, respectively, were Cu deprived according to diagnostic criteria for *Bos taurus* cattle. High hepatic Mo concentrations in 17% of cases possibly reflected excessive dietary Mo intake. Selenium was too low in 92% and 80% of cattle, whereas plasma Mn concentrations were largely below the detection limit. Plasma Cu concentrations were lower in cattle grazing at lowest versus those grazing at the highest altitude and were also lower in lean to medium cattle than in more fat inidividuals. No differences in hepatic mineral concentrations were detected between cattle with different types of parasitic infection. In conclusion, bovine mineral deficiencies were present in the Gilgel Gibe area and were associated with grazing altitude and body condition score.

Considering the highly prevalence of Cu deficiency, and the role of S as an Cu antagonist, effective assessment of the status of this element is important yet remains difficult. Rumen fluid S concentrations are preferred, but are difficult to sample under practical conditions. In **Chapter 3**, the objective was to evaluate salivary S concentration as estimator of S status in cattle. Saliva and rumen fluid samples were collected from dairy cows (n = 16) with a rumen fistel as well as samples of different feedstuffs offered to the animals and N and S concentrations were determined. Salivary S concentrations were not found to be predictive for rumen fluid or dietary S concentrations. The log transformed salivary N:S ratio could linearly predict the rumen fluid N:S ratio but left too much residual variation to serve as a reliable predictor.

The next study, presented in **Chapter 4**, aimed to identify the early effects of dietary trace elements on anti-oxidant status and performance. Compared to the control group, in adult grass-fed zebu bulls (n = 8) receiving trace element supplementation (Zn, Mn, Cu, Se, I and Co) during four weeks, plasma Cu, Zn, Mn and Se concentrations and faecal Cu, Zn, Mn and Se were increased. On the contrary, trace element supplementation did not affect anti-oxidant status, namely plasma ceruloplasmin and superoxide dismutase activities. Also, no effects on apparent nutrient digestibility, apparent trace element absorption (except for Se and I) or plasma acyl carnitines (indicators of available energy substrates) were observed. Overall, despite clear improvement in trace element status, supplementation did not affect anti-oxidant status, nutrient digestibility or utilisation in grass-fed zebu beef cattle.

Subsequently, the effect of trace element supplementation under practical farming conditions on bovine trace element status and milk concentrations as well as anti-oxidant status, immune response and performance was investigated (**Chapter 5**). At small-scale urban dairy farms (n = 16) with crossbred (zebu × taurine) cows, Cu (17 %) and marginal Se (30 %) deficiencies were present and measures for oxidative stress were associated with plasma Fe concentrations. In a double-blinded intervention study, cows on farms enrolled in a trace element supplementation (Cu, Zn, Se, Co and I) programme for 150 days, experienced a bigger increase in plasma Se and Cu and milk Se, coinciding with a larger decrease of plasma Fe concentrations. None of the parameters for antioxidant status and immune response nor of performance were affected by treatment. In the supplemented group, body condition scores of cows even decreased more than in the control group. In conclusion, despite improved Cu and Se status and Se concentrations in milk, cows on tropical urban dairy farms did not seem to benefit from trace element supplementation, with respect to the parameters investigated.

Afterwards, **Chapter 6** studied the trace element distribution in edible bovine tissues, as these are of importance both for animal health and human nutrition. Adult zebu bull semitendinosus and cardiac muscle, liver and kidney (n = 60) and plasma (n = 28) were sampled at the local abattoir. Deficient levels of Cu were found in plasma, livers, kidneys and semitendinosus muscles in addition to the suboptimal Se concentrations observed in plasma and semitendinosus muscles and high levels of Fe in semitendinosus muscles.

Trace elements were mainly stored in the liver and cardiac muscles generally contained higher concentrations of trace elements than semitendinous muscles. A strong association was found between liver and kidney concentrations of most elements, while on the contrary, for few elements (Co, Se), liver storage correlated well with storage in muscle. Plasma concentrations of Cu, Se, Co were well related with their respective liver concentrations and for Co and Se, also with cardiac muscle concentrations. As previously shown, multiple trace element deficiencies were present in zebu cattle in the area, and these affected tissue concentrations.

In the last research chapter, namely **Chapter 7**, the objective was to investigate potential differences in trace element metabolism in zebu and crossbred cattle. Throughout an eleven weeks trial, feeding zebu and zebu × Holstein Friesian cross heifers (n = 16) a Cu deficient grass-diet supplemented with the Cu antagonist Mo, zebu cattle maintained initial plasma Cu concentrations just below the threshold for deficiency, whereas crossbred cattle gradually became severely Cu deficient. In contrast, at the onset of the trial, plasma Co and Zn were higher in zebu cattle, but evolved to values similar to those in crossbred cattle. At the end of the trial, kidney Se; liver Fe and Cu; kidney and cardiac muscle Co concentrations were significantly higher in zebu than in crossbred heifers. Increased hepatic mRNA expression of selected Cu-regulatory proteins and Cu-related enzymes was significantly greater in zebu than in crossbred cattle. In contrast, duodenal mRNA expression of such compounds was not found to differ between types of cattle. Above mentioned data point to the possibility of a more efficient use of dietary Cu in zebu as compared to zebu × taurine crossbred cattle resulting in higher sensitivity to Cu deficiency in *B. taurus* influenced cattle.

Finally, in the **General Discussion**, the obtained insights from the research chapters were compared with current scientific literature and all data were compiled in order to gain a broader perspective on the raised research questions.

Overall, we concluded that the trace element supply for zebu-influenced cattle in an exemplary tropical region was imbalanced and related to plant, environment and management factors. Concomitantly, low trace element concentrations were found in bovine transport and storage pools, which associated with age, sex, body condition

score and type of cattle (zebu versus crossbred). The urgent need for refinement of current requirements and threshold values both in zebu and taurine cattle was emphasised. Linear regression analysis (Co and Se) as well as the receiver operator characteristic method (Cu) showed that plasma was useful to predict liver status. In these cattle, trace element supplementation effectively raised concentrations in the transport pool but performance and production did not benefit from this, the latter suggesting a significant role of management. However, trace element status did affect trace element distribution and secretion and therefore human nutrition, although with respect to the latter, concentrations were variable and averages should be employed with care.

Samenvatting

Wereldwijd is grazend vee gevoelig voor tekorten aan sporenelementen. In de **Inleiding** van dit werk werd de huidige kennis over sporenelementen in rundvee besproken. In een ideale wereld kunnen sporenelementen, "geschenken uit de natuur", vrij bewegen doorheen de bodem-plant-dier cyclus. Een dergelijk evenwicht wordt echter zelden waargenomen en in weerwil van eerder onderzoek, werden nog niet alle factoren die de stroom aan sporenelementen beïnvloeden, en op deze manier het bestendigen van een optimale sporenelementen status in runderen belemmeren of faciliteren, in kaart gebracht. Bovendien is er weinig geweten over de brede impact van sporenelementenstatus in runderen. Zo zijn bijvoorbeeld de gerapporteerde effecten van verschillen in sporenelementstatus op immuniteit en productie tegenstrijdig. Bovenden werden deze effecten tot heden, zelden bestudeerd onder praktijkomstandigheden. Daarnaast wordt de invloed van de sporenelementenstatus op concentraties aan sporenelementen in eetbare weefsels en melk, potentieel belangrijk voor de menselijke aanvoer van sporenelementen, niet goed begrepen. Bovendien is er weinig onderzoek verricht naar sporenelementen in zebu-vee, de dominante vee-soort in tropische gebieden, desondanks er in deze gebieden een hoger risico voor onevenwichten bestaat. Daarom vormden deze aspecten de belangrijkste aandachtspunten van dit proefschrift. Om de geformuleerde onderzoeksdoeleinden volledig te onderzoeken werd het Gilgel Gibe stroomgebied, in Zuid-West Ethiopië, geselecteerd als onderzoekssite.

Hoofdstuk 1 onderzocht het gehalte aan sporenelementen in diëten van grazende runderen en het effect van plantenfactoren, milieu en management op dit gehalte. Planten geconsumeerd door grazende zebu-vee kuddes (n = 19) werden bemonsterd en de totale diëtaire inname aan sporenelementen werd geschat door middel van monitoring van planten-opname. Omgeving en management waren intens verbonden in deze kuddes, met het typisch voorkomen van gezamenlijk grazen in grote kuddes over langere afstanden op Planosol-Vertisol (PV) bodemtypes, op lagere hoogte. De bemonsterde planten Cu bevatten matige Cu (72 %), Se (59 %) en Zn (43 %) concentraties, en de samengestelde diëten bevatten ook vaak nadelige Cu:Mo (19 %) en Fe:Cu (41 %) verhoudingen. Over het algemeen bevatten kruidachtige en houtachtige

planten grotere hoeveelheden sporenelementen dan grassen en gewasresten (bv. Cu en z'n belangrijkste antagonist, S), terwijl voor andere elementen, plantenconcentraties verschilden volgens de hoogte van het graasgebied (bv. Mn en Zn). Bovendien was de hoeveelheid aangevoerde sporenelementen in planten geassocieerd met bodemtype; planten groeiend op Nitisol-Acrisol-Ferralsol bodem types bevatten nl. meer Fe dan die op PV bodems. Kuddes die op PV bodems graasden hadden een hogere inname van Cu en Fe maar minder Mo dan die op NAF bodem. Gemeenschappelijk grazende kuddes namen hogere gehaltes aan Mo en Mn op dan individueel grazende en de afgelegde afstand tijdens het grazen had een positief effect op diëtar Mn, terwijl het supplementeren van het dieet met gewasresten het diëtaire Zn- en Se-gehalte negatief beïnvloedde. Omgeving en bepaalde managementsstrategiëen waren dus nauw verbonden, en kunnen zowel de verklaring als de oplossing bieden voor de belemmerde aanvoer van sporenelementen.

Hoofdstuk 2 richtte zich vervolgens op het evalueren van de mineralenstatus in grazend zebu-vee en het blootleggen van geassocieerde factoren. Analyses van plasma- en leverstalen bemonsterd in volwassen zebu-vee grazend in het stroomgebied (n = 90) en zebu-stieren geslacht in het lokale abattoir (n = 53) respectievelijk, brachten een Cu-tekort in het gebied aan het licht, aangezien respectievelijk 68 % en 47 % van het vee, gedepriveerd waren aan Cu. De hoge lever Mo-concentraties in 17 % van de gevallen waren mogelijks een uiting van overmatige Mo inname. De Se status was te laag in 92 % en 80 % van de respectievelijk bemonsterde runderen, terwijl plasma Mn-concentraties zich grotendeels onder de detectielimiet bevonden. Plasma Cu-concentraties waren lager bij runderen grazend op lagere dan op de hoogste graslanden en waren ook lager in mager tot normaal vee dan in vettere individuen. Er werden geen verschillen in lever mineralenconcentraties geobserveerd tussen runderen met verschillende parasitaire infecties. In conclusie, kan er gesteld worden dat tekorten aan mineralen aanwezig waren in vee van het Gilgel Gibe gebied en dat deze tekorten geassocieerd waren met graashoogte en conditiescore.

Gezien de hoge prevalentie aan Cu-deficiëntie in vee, en de rol van S als Cu-antagonist, is een effectieve beoordeling van de dierlijke status van dit element belangrijk. Zwavelconcentraties in de pensvloeistof genieten de voorkeur als schatter, maar zo'n

stalen zijn moeilijk te verkrijgen onder praktijkomstandigheden. **Hoofdstuk 3** beoogde het evalueren van S-concentraties in speeksel als schatter van S-status in rundvee. Concentrations aan N en S werden bepaald op speeksel- en pensvloeistofmonsters verzameld in melkkoeien (n = 16) met een pensfistel, evenals op monsters van de verschillende voedingsmiddelen aangeboden aan de dieren. We konden niet aantonen dat speeksel S-concentraties een voorspellende waarde hadden voor S-concentraties in pensvloeistof of dieet. De log-getransformeerde N:S verhouding in speeksel kon de N:S verhouding in de pensvloeistof lineair voorspellen maar deze verhouding vertoonde te veel residuele variatie om op dit moment ingezet te worden als betrouwbare schatter.

De volgende studie, gepresenteerd in **Hoofdstuk 4**, had tot doel de vroege effecten van diëtaire sporenelementen op anti-oxidant status en prestaties te onderzoeken. Vergeleken met de controlegroep, hadden volwassen gras-gevoederde zebu-stieren (n = 8) gedurende vier weken gesupplementeerd met sporenelementen (Zn , Mn , Cu , Se , I en Co), hogere plasma Cu-, Zn-, Mn- en Se- en fecale Cu-, Zn-, Mn- en Se-concentraties. Daarentegen werden geen effecten van supplementatie op anti-oxidant status nl. plasma ceruloplasmine en superoxide dismutase activiteiten, geobserveerd. Ook werden er geen effecten waargenomen op de schijnbare verteerbaarheid van het dieet, op de schijnbare sporenelementenabsorptie (behalve voor Se en I) of plasma acylcarnitines (dewelke indicatoren zijn voor beschikbare energiesubstraten). Ondanks duidelijke verbetering van de sporenelementenstatus leek supplementatie dus geen invloed te hebben op anti-oxidant status en diëtaire verteerbaarheid of benutting in grasgevoerderde zebu-runderen.

Vervolgens werd onder praktijkomstandigheden het effect onderzocht van sporenelementen supplementatie op runderstatus en melk concentraties onderzocht alsook op anti-oxidant status, immuunrespons en prestaties (**Hoofdstuk 5**). Op kleinschalige stedelijke melkveebedrijven (n = 16) met gekruiste (zebu × taurine) runderen, waren tekorten aan Cu (17 %) en marginale tekorten aan Se (30 %) aanwezig. Tevens waren parameters voor oxidatieve stress geassocieerd met plasma Fe-concentraties. In een dubbel-blinde interventiestudie, ervaarden runderen afkomstig van bedrijven die gedurende 150 dagen deelnamen aan een sporenelementen (Cu, Zn, Se, Co en I) supplementatie-programma, een grotere toename van de plasma Se- en Cu-

en melk Se-concentraties, samenvallend met een grotere afname van plasma Fe concentraties. Geen van de parameters voor antioxidant status, immuunrespons of productie werden beïnvloed door de supplementatie. In de gesupplementeerde groep gingen lichaamsconditie-scores zelfs sterker achteruit dan in de controlegroep. Ter conclusie stellen we, ondanks een verbeterde Cu- en Se-status en Se-gehaltes in melk, dat runderen op tropische melkveebedrijven niet leken te profiteren van sporenelementensupplementatie, met betrekking tot de onderzochte parameters .

Daarna werd in **Hoofdstuk 6** de distributie aan sporenelementen in eetbare weefsels van zebu-runderen bestudeerd. Dit speelt zowel voor de gezondheid van dieren en menselijke voeding een belangrijke rol. Van volwassen zebu stieren werden semitendinosus- en hartspier, lever, nier (n = 60) en plasma (n = 28) bemonsterd in het plaatselijke slachthuis. Deficiënte niveaus aan Cu werden gevonden in plasma, lever, nier en semitendinosus spier. Hiernaast werden suboptimale Se concentraties waargenomen in plasma en de semitendinosus spier en een hoog niveau aan Fe in de semitendinosus spier. Sporenelementen werden voornamelijk opgeslagen in de lever en de hartspier bevatte over het algemeen hogere concentraties aan sporenelementen dan de semitendinosus spier. Voor de meeste elementen werd een sterke associatie gevonden tussen lever- en nierconcentraties, terwijl daarentegen van slechts enkele elementen (Co , Se), de leveropslag goed correleerde met de opslag in de spieren. Plasmaconcentraties aan Cu, Se and Co waren goed gecorreleerd met hun respectievelijke leverconcentraties en voor Co en Se, ook met hartspiergehaltes. Zoals eerder aangegeven, waren er meerdere tekorten aan sporenelementen in zebu runderen in het gebied. Deze tekorten beïnvloedden de weefselconcentraties.

In het laatste onderzoekshoofdstuk, nl. **Hoofdstuk 7**, was het onderzoeken van mogelijke verschillen in sporenelementen metabolisme in zebu en gekruiste runderen het doel. Over een elf weken-durende proef, waarin zebu en gekruiste zebu × Holstein Friesian vaarzen (n = 16) aan een Cu deficiënt grasdieet aangevuld met de Cu -antagonist Mo, blootgesteld werden, onderhielden de zebu vaarsen hun plasma Cu-concentraties net onder de drempel voor deficiëntie, terwijl gekruiste vaarzen langzamerhand ernstig Cu-depriveerd werden. Echter, bij het begin van de proef, waren plasma Co en Zn hoger in zebu runderen maar de concentraties evolueerden tot waarden vergelijkbaar met die in

gekruiste runderen. Aan het einde van de proef, waren nier Co- en Se-, lever Fe- en Cu- en hartspier Co concentraties significant hoger in zebu- dan in gekruiste vaarzen. De verhoogde lever mRNA expressie van geselecteerde Cu-regulerende eiwitten en verwante enzymen was ook significant hoger in zebu- dan in gekruiste vaarzen terwijl de duodenale mRNA expressie niet verschilde tussen de rundertypes. Bovenstaande gegevens wijzen op de mogelijkheid van een efficiënter gebruik van diëtair Cu in zebu tegenover zebu × taurine vee, resulterend in een hogere gevoeligheid voor Cu deficiëntie in runderen met een taurine invloed.

Tenslotte werden in de **Algemene Discussie**, de verkregen inzichten uit de onderzoekshoofdstukken vergeleken met de huidige wetenschappelijke literatuur en alle gegevens gecompileerd met het oog op het verkrijgen van een breder perspectief op de vooraf geformuleerde onderzoeksvragen.

We concludeerden dat de aanvoer aan sporenelementen voor zebu-beïnvloed vee, in een tropische voorbeeldregio, onevenwichtig is en geassocieerd met plantaardige, omgevings- en managementsfactoren. Tergelijkertijd werden lage gehaltes aan sporenelementen gevonden in de transport- en opslag pool van runderen, die in verband stonden met leeftijd, geslacht, conditiescore en type vee (zebu versus kruising). De dringende behoefte aan verfijning van de huidige sporenelementen vereisten en diagnostische drempelwaarden zowel in zebu en taurine vee werd benadrukt. Uit lineaire regressieanalyse (Co en Se) en de receiver operator characteristic methode (Cu) bleek dat plasma nuttig was om de leverstatus voorspellen. Sporenelementen supplementatie verhoogde de concentraties in de transport pool, maar de prestaties en de productie leken niet te zijn beïnvloed, hetgeen kan duiden op een belangrijke rol van management. Daarentegen beïnvloedde sporenelementen supplementatie wel degelijk de sporenelementen distributie en secretie en dus menselijke voeding, hoewel, met betrekking tot dit laatste, concentraties variabel waren. Er dient dus met zorg met gemiddelde waarden moet omgesprongen worden.

Curriculum Vitae

Veronique Dermauw werd geboren op 25 mei 1985 te Kortrijk. In 2003 behaalde zij met onderscheiding haar diploma secundair onderwijs in de richting Latijn-Wetenschappen aan het O.L.V.H.-Instituut te Waregem en aansluitend startte zij haar studies Diergeneeskunde aan de Universiteit Gent. In 2009 behaalde zij het diploma Dierenarts (Optie Onderzoek) met onderscheiding.

In oktober 2009 trad zij in dienst aan de vakgroep Voeding, Genetica en Ethologie en in januari 2010 behaalde zij een IWT-specialisatiebeurs. Gedurende vier jaar deed zij aan deze vakgroep onderzoek naar de sporenelementenstatus van Ethiopische zebu (*Bos indicus*) runderen en het effect daarvan op diergezondheid. Ze is lid van het IUC-JU project van VLIR-UOS en organiseerde tijdens haar verblijven in het buitenland meerdere workshops en informatiesessies. Ze was ook intensief betrokken bij onderzoek naar de sporelementenvoorziening in biologisch melkvee in Vlaanderen (ADLO-project). Tevens begeleidde ze meerdere studenten bij hun afstudeerwerk en voorbereiding op hun stage in Ethiopië.

Veronique Dermauw is auteur en medeauteur van meerdere publicaties in internationale wetenschappelijke tijdschriften en was meermaals spreker op internationale congressen.

Veronique Dermauw was born on May 25, 1985 in Kortrijk. In 2003 she obtained her diploma of secondary education in Latin-Sciences with distinction at the OLVH-Institute in Waregem. Consequently, she started her studies in Veterinary Medicine at Ghent University. In 2009, she graduated as Veterinarian (Option Research), with distinction.

In October 2009 she joined the Department of Nutrition, Genetics and Ethology, and she obtained an IWT scholarship in January 2010. At this department, she investigated the trace element status of Ethiopian zebu (*Bos indicus*) cattle and its effect on health. She is a team member of the IUC-JU project of VLIR-UOS and organized several workshops and information sessions during her stay abroad. She was also closely involved in research on the trace element supply in organic dairy cattle in Flanders (ADLO project). She supervised several students during their thesis work and when preparing for research in Ethiopia.

Veronique Dermauw has authored and co-authored several publications in international scientific journals and was repeatedly speaker at international conferences.

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&

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